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14. ABSTRACT

The most significant research findings in this time period include the fact that we have demonstrated that our genetically-modified pigskin grafts will perform as well human cadaveric allogeneic skin grafts as a temporary biologic cover for severe burn injuries for war-fighters. Our xenoskin transplants from pig to baboons have confirmed that our genetically-modified pigskin grafts last as long as baboon allograft skin, are not damaged by freezing, and are effective at treating full-thickness skin injuries typically seen in burn injuries sustained in combat. In addition, a short course of immunosuppression may enhance the efficacy of these grafts. Finally, our in vitro analysis has suggested a number of immunologic targets that may improve this approach in particular and xenotransplantation in general.

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INTRODUCTION

The subject of this project involves "Wound infection and healing" – with special reference to "New treatment protocols, drugs, biologics, and devices to reduce wound-related infections and accelerate wound healing." In particular, we are attempting to validate the effectiveness of a novel treatment for severe burns using a genetically-modified porcine skin graft. The **purpose** of this project is to determine whether genetically-modified pig skin grafts will perform as well as or better than human cadaveric allogeneic skin grafts as a temporary biologic cover for severe burn injuries [1-4]. Since human cadaveric allogeneic skin grafts are the current "gold standard" for temporary skin grafts [5;6] and since our genetically-modified pig skin would have significant advantages related to availability, cost, safety and ethical considerations [7-10] the **scope of the research** will be to develop this new product to serve as skin grafts for the initial treatment of severe battlefield injuries, including burns and other causes of significant skin loss.

BODY

This section of the Year 1 of 3 Annual Report describes the research accomplishments associated with the tasks/specific aims described in our approved Statement of Work, in particular, with regard to Specific Aim 1. To date, we have performed 50 grafts in the first 2/3 of Year 1. As such, we are on schedule with regard to completion of the project.

Specific Aim 1: Compare the survival of skin grafts to baboons from GalT-KO swine to the survival of skin grafts from unmodified swine or from allogeneic baboons and study the response of these grafts by gross examination, by histology and evaluation of the cellular and humoral immune responses evoked.

Task 1. Develop and Compare survival of skin grafts to baboons from GalT-KO swine:

1a. Evaluate effect of immunosuppression on outcome
1b. Evaluate role of graft technique to outcome
1c. Evaluate effect of skin treatment and storage

Clinical and Histopathologic Data

Confirmation of Preliminary Data and Reproducibility of Xenotransplantation Model: Our first set of experiments was designed to replicate the experimental conditions of those described in our preliminary data (pg. 13, Appendix 1) and to begin to collect data for Tasks 1a and 1b. Two baboons, B266 and B267, were transplanted with the scheme shown on pg. 17, Appendix 2, slide 1. Split-thickness recipient wounds were prepared with a dermatome, and the fresh, split-thickness grafts from the 4 skin sources (**Self baboon**, Allogeneic baboon, **Gal+** swine and **GalT-KO** swine) were placed. The only change we made involved placing a border of normal skin between grafts to avoid the local infections observed in our initial experiment, which may have affected the outcome and/or clinical appearance of the graft immediately adjacent. B266 received no immunosuppression, while B267 received cyclosporine. The dosing regimen was based on the cyclosporine (CyA) regimen that we have used successfully for induction of long-term survival of renal allografts across a class I allogeneic barrier [11].

The results confirmed our initial findings and clearly demonstrated the reproducibility of this xenotransplantation model. In both B266 (no immunosuppression) and B267 (CyA), the Gal+ skin was rejected within 4 days, appearing as a "white graft" that had not vascularized (pg. 18, Appendix 2, slide 2, yellow arrow). By contrast, in baboon B266, the GalT-KO skin graft was still viable at postoperative day 7, similar to the allogeneic skin graft (pg. 18, Appendix 2, slide 2, red arrows). Both were beginning to show signs, both by gross inspection and histopathologically, of rejection, and by day 11, both were rejected.

Results were similar for B267, with possibly a slightly extended time course for survival of both allogeneic and GalT-KO skin grafts, likely due to the effect of the cyclosporine. (pg. 19, Appendix 2, slide 2, red arrows). By day 11, both were rejected, however, both clinically and histologically. Histology exhibited healthy dermis and epidermis on self skin (pg. 19, Appendix 2, slide 4, yellow arrow), and complete vacuolization of dermis- consistent with complete rejection- of Gal+ skin (pg. 20, Appendix 2, slide 4, red arrow), by POD 7. In contrast, early rejection of both GalT-KO (pg. 20, Appendix 2, slide 5, yellow arrow), and Allo (pg. 21, Appendix 2, slide 5, red arrow) grafts were observed at POD 7, both of which showed a marked lymphocyte infiltrate in the dermis. The delay in rejection of GalT-KO grafts due to the presence of CyA is evident histologically when baboons B266 (no Cy A) and B267 (CyA) are compared at POD 7: B266 shows a lymphocytic infiltrate in the dermis and a sloughed epidermis (pg. 22, Appendix 2, slide 6, yellow arrow), while B267 shows an intact epidermis and dermis with only a modest lymphocytic infiltrate (consistent with *early* rejection) (pg. 22, Appendix 2, slide 6, red arrow).

Investigation of Possible Confounding Effects of More Than One Graft Type: The next experiments were designed to address an important question involving Tasks 1a, b, and c: that it is possible- though unlikely- that rejection of a Gal+ skin graft, or even of an allogeneic baboon skin graft, could influence the survival of the GalT-KO skin graft on the same animal. We evaluated clinical, histopathological and in vitro immunologic data on the next two animals to examine whether there was evidence to support this hypothesis (and therefore require a single-graft-per-animal approach rather than a more efficient 4-grafts-per-animal-approach, which also provides an advantage of an internal control both clinically and immunologically).

We utilized the following schematic for the next two baboons, B268 and B269 (pg. 23, Appendix 3, slide 1). Note that, by not grafting Gal+ skin onto this baboon, we eliminated the possibility of a clinical immunologic effect on the GalT-KO graft secondary to increased inflammatory mediators that may occur from the rejection of a Gal+ graft in the same individual baboon. We found no such effect: the GalT-KO and Allo skin grafts behaved similarly to the previous two baboons, again both surviving intact until day 7 (pg. 24, Appendix 3, slide 2, red arrows).

Conversely, in animal B269, we tested the effect of Gal+ rejection on the baboon's immunologic response to the Allogeneic graft. Again, we found no such effect: the Allo skin graft survived intact until day 7 (pg. 25, Appendix 3, slide 3, red arrow). These results suggested that a more efficient, 4-grafts-per-animal approach might allow more rapid accumulation of data with more efficient use of animals. In addition, the self and Gal+ grafts would provide an advantage of an internal control in each animal both clinically and immunologically.

Confirmation of Second-Set Rejection, Processing of Frozen Grafts, Grafting on Full-Thickness Wound Beds: The next set of experiments was designed to address an important question involving Tasks 1b, and c, i.e: Would humoral sensitization and rejection proceed identically for a second set of skin grafts of Allo and GalT-KO grafts given to a previously grafted baboon (i.e. *regrafting* baboons B266 and B267)? In other words, what is the relative sensitization potency of Allo vs. non-Gal antigens once they have been "seen" by a primate's immune system? The answer to this question would have potential implications for clinical use of the GalT-KO skin grafts if a second graft was necessary on a burned soldier, and suggests another possible use for GalT-KO skin- a combination approach- when cadaver skin is also available to treat a soldier. For example, if a second graft was necessary for a burned soldier, after an initial cadaver graft had been placed, knowledge that the patient would be not have been sensitized to non-Gal antigens by the cadaver skin graft- a phenomenon that has been demonstrated for solid organ xenotransplantation [12]- would allow use of GalT-KO skin *after* the cadaver graft sloughs (or vice versa), thereby buying time for the metabolic recovery of the patient prior to definitive wound coverage. This treatment algorithm has significant potential for improved outcomes in severe burns.

In addition, we utilized these animals as an opportunity to acquire early data regarding two other variables, processing of frozen skin grafts, and grafting on a full-thickness wound bed, both of which are important military practicalities. We utilized the schematic shown on pgs. 26-27, Appendix 4, slides 1 and 2, for regrafting of baboons B266 and B267. We observed rapid rejection of both Allo and GalT-KO second-set skin grafts, with no difference in pace of rejection. Grafts were rejected by day 4 (pgs. 28-29, Appendix 4, slides 3, 4). We noted no difference between fresh and frozen grafts in terms of early take, and graft take was excellent on a full thickness wound bed (evidenced by self grafts) (pgs. 28-29, Appendix 4, slides 3, 4). Determination of whether sensitization to Allo affects survival of a subsequent GalT-KO skin graft, and vice versa, is in progress.

Frozen Grafts and Full-Thickness Wound Beds: To confirm that the processing of frozen grafts has no effect on GalT-KO skin viability, and that GalT-KO skin grafts will take on full-thickness wound beds as well as split-thickness, we performed autografts on GalT-KO pigs (i.e. pig-to-self-pig) on both full and split-thickness wound beds comparing fresh and frozen skin (Tasks 1b and c). We performed these experiments to assure that the rapid rejection of second-set grafts was due to sensitization and not to technical factors regarding our freezing protocol or wound bed preparation (theoretically, grafts should take equally well on split or full thickness wound beds). We observed no differences, as all grafts healed successfully. Appendix 5 (pg. 30) shows viable fresh and frozen autologous grafts on split thickness beds intraoperatively, and on PODs 0, 1, 3, 8, and 14 (pgs. 30-34, slides 1-5), as well as viable fresh and frozen autologous grafts on *full-thickness* wound beds on PODs 0, 3, 7, 23, and 51 (pgs. 35-40, slides 6-11).

Xenoskin Transplantation on Full-Thickness Wound Beds: The next set of experiments was designed to replicate the experimental conditions of those diagrammed on pg. 17, Appendix 2, slide 1, however on *full-thickness* wound beds, representative of injuries requiring grafting in the field (Tasks 1a,b,and c). Two baboons, B280 and B282, were transplanted with the schematic described on pg. 41, Appendix 6, slide 1. Full-thickness recipient wounds were created with a scalpel, and the different, fresh, split-thickness grafts from the 4 skin sources (**Self** baboon, **Allogeneic** baboon, **Gal+** swine and **GalT-KO** swine) were placed. B280 received no immunosuppression, while B282 received cyclosporine.

The results of these experiments were consistent with our initial findings on split-thickness wound beds and clearly demonstrated the reproducibility of our xenotransplantation model in clinically-relevant *full-thickness* wounds. In both B280 (no immunosuppression) and B282 (CyA), the Gal+ skin was rejected within 4 days, appearing as a "white graft" that did not vascularize (pgs. 42-43, Appendix 6, slides 2 and 3, yellow arrows). By contrast, in baboon B280 the GalT-KO skin graft was viable at postoperative day 7, similar to the allogeneic skin graft. Both were beginning to show signs of rejection both visibly and histopathologically, and by day 11, both were rejected, as previously found (pg. 42, Appendix 6, slide 2, red arrows). Results were similar for B282, in which both the GalT-KO and allogeneic skin were clinically viable at postoperative day 7 (pg. 43, Appendix 6, slide 3, red arrows). In vitro analysis is currently underway in these experiments.

Xenoskin Transplantation of Fresh vs. Frozen Grafts on Full-Thickness Wound Beds: We next examined fresh vs. frozen grafts on full-thickness wound beds (Tasks 1b and c). Two baboons, B283 and B285, were transplanted according to the schematic described on pg. 44, Appendix 7, slide 1. Full-thickness recipient wounds were created with a scalpel, and the different, fresh or frozen split-thickness grafts (that had been previously harvested and frozen one week preoperatively) from the 4 skin sources (**Self** baboon, **Allogeneic** baboon, **Gal+** swine and **GalT-KO** swine) were placed. Neither B283 nor B285 received immunosuppression. The results again demonstrated that both fresh and frozen xenografts and allografts enjoyed comparable survival on full thickness defects. Results were similar for B283 and B285, in which both the fresh and frozen GalT-KO and allogeneic skin grafts were clinically viable at postoperative day 7. Control self grafts showed 100% acceptance and survival and Gal+ grafts again failed to engraft, appearing as "white grafts". In vitro analysis is also underway in these experiments.

Summary of Clinical and Histopathologic Data

We have de monstrated that 1) GalT -KO skin xenotransplants from pi g-to-baboon last at least as long as baboon allogeneic skin transp lants; 2) second-set graf ts reject rapidly, typical of a sensitized imm une response; 3) graft surviv al is unaffected by freezing/thawin g; and 4) graft survival is com parable on partial-thickness and full-thickness recipient wound beds.

a) Baboons B266, B267 (1st grafting): (pgs. 17-22, Appendix 2)

- GalT-KO and Allo both rem ained intact until rejection between POD 7 and 11, per clinical and histologic findings
- Cyclosporine prolonged survival of both GalT-KO and Allo grafts
- Controls: Self (no rejection) and Gal+ skin (the Gal+ graft rejected in a hyperacute fashion, as predicted: the Gal+ g raft was a "white graft" by POD4, sugges ting that it never vascularized because th e vasculature was destroyed due to naturally-pre sent, pre-form ed anti-Gal antibodies in the baboon that initiated the com plement cascade and endothelial cell destruction)

b) Baboons B268, B269: (pgs. 23-25, Appendix 3)

- GalT-KO and Allo rejected between POD 7 and 14
- Gal+ skin did not affect the survival of GalT-KO or Allo skin
- Controls: Self (no rejection) and Gal+ skin (POD 4 white graft again)

c) Baboons B266, B267 ("2nd set" grafting (Regrafting following sensitization by the first set of grafts)): (pgs. 26-29, Appendix 4)

- GalT-KO and Allo rejected by POD 4 (more quick ly than in the f irst set in which they rejected somewhere between POD 7 and 11
- Neither frozen/thawed grafts nor full thickness wound beds detrim entally effected early survival of the grafts
- Cyclosporine had no effect on the 2nd set rejection time of both GalT-KO and Allo grafts

d) Pig-to-pig Split-thickness wound beds, fresh vs. frozen GalT-KO autografts: (pgs. 30-34, Appendix 5)

- Freezing and thawing of grafts did not detrimentally affect survival of the autografts

e) Pig-to-pig Full-thickness wounds beds, fresh vs. frozen GalT-KO autografts: (pgs. 35-40, Appendix 5)

- Neither freezing and thawing nor use of full thickness w ound be ds detrim entally affected survival of grafts

f) Baboons B280, B282: (pgs. 41-43, Appendix 6)

- Full thickness wound beds did not detrimentally affect survival of the xenogeneic Galt-KO skin grafts

g) Baboons B283, B285: (pg. 44, Appendix 7)

- Freezing an d thawing o f grafts did not detrim entally affect survival of the xenogen eic Galt-KO skin grafts

In Vitro Data Analysis

1) We have demonstrated that baboon recipients of skin grafts from swine that express the Gal antigen (i.e. Gal+ swine) reject Gal+ skin grafts in a hyperacute manner, presumably due to preformed antibodies (B cell response to primary grafting). Antibody assays were consistent with this hypothesis. For example, ELISA data (pg. 45, Fig. 1, Appendix 8) showed the presence of pre-formed anti-**Gal** IgM (top panel) and IgG (bottom panel) antibodies in baboon B266. A similar result was seen for baboon B267 (pg. 46, Fig. 2, Appendix 8), and Gal+ skin grafts on both baboons led to white grafts.

2) Conversely, examination of B cell responses by analyzing FACS data showed that neither anti-**Allo** IgM or IgG antibodies nor anti-**nonGal** IgM or IgG antibodies (anti-nonGal antibodies = baboon antibodies to swine antigens other than the Gal moiety) were present pretransplant in the baboon (pg. 47, Fig. 3, Appendix 8). FACS data also showed that **Allo** antibody levels did not rise significantly after **Allo** skin transplantation (pg. 48, Fig. 4, Appendix 8) - a finding consistent with the expected T cell-mediated mechanism of allotransplantation rejection rather than B cell/antibody-mediated rejection.

3) T-cell responses involved in primary baboon responses to **Xeno** vs. **Allo** skin grafts were suggested by in vitro study with the **Mixed Lymphocyte Reaction (MLR):** After the 1st skin grafts in B266 and B267, MLR assays showed a *pre* skin transplant **Allo** T cell response stronger than the **Xeno** T cell pre skin transplant response (pg. 49, Fig. 5, Appendix 8). The **Xeno** T cell response was stronger than the **Allo** response *post* skin grafting (POD 14, POD 21), however. These results are consistent with known relative T cell responses of allogeneic and xenogeneic transplantations.

4) In baboon B267, which was treated with Cyclosporine A, the CyA suppressed both **Allo** and **Xeno** T cell responses, as expected. This suppression in T cell reactivity can be observed by comparing the counts per minute (cpm) for the responses at each of the time points, which have lower cpms (see red circles around cpm values on pgs. 49-50, Figs 5 and 6, Appendix 8).

5) With regard to **T-cell responses** involved in secondary baboon responses after 2nd-set skin grafting (i.e. post-sensitization), **Xeno** T cell responses were stronger than **Allo**, and CyA had little effect (the CyA dose may be too low to suppress a sensitized T cell response) (pgs. 51-52, Figs. 7 and 8, Appendix 8).

6) Clinical observation of more rapid rejection of second-set grafts suggested sensitization by the first set of grafts. Gal+ grafts rapidly rejected again, similar to the first set, but now both the GalT-KO and Allo grafts also rapidly rejected, consistent with presensitization due to the previous nonGal swine and Allo baboon antigens "seen" at the time of first grafting (Allo second-set grafts were from the original individual Allo donor baboons that donated first-set skin grafts). It appeared that the rejection of GalT-KO second-set grafts were rejected by an elevated B cell response, while second-set rejection of Allo grafts exhibited a less pronounced B cell response, a finding consistent with known mechanisms of T cell sensitization of second-set alloreactivity. FACS analysis supported these hypotheses. For example, FACS analysis of anti-nonGal IgG peaked at POD 7 after second-set GalT-KO grafts vs. POD 14 for first-set GalT-KO grafts (pg. 53, Fig. 1, Appendix 9). This effect was present, yet less pronounced for anti-Allo antibodies, nevertheless, the second-set Allo grafts rapidly rejected, suggesting a T cell contribution. These hypotheses are under further investigation in our laboratory.

7) We have demonstrated, with baboon B268 (self, GalT-KO, allo, CyA), that pre-transplant the Allo T-cell response was again found to be greater than the Xeno response (consistent with our baboon B266, B267 data) (pg. 54, Fig. 1, Appendix 10). However, post-transplant Xeno response increased and was similar to the Allo response by POD 30 (pg. 55, Fig. 2, Appendix 10).

8) We have demonstrated that baboon B268 exhibited a similar B-cell response to baboons B266 and B267. For example, FACS data exhibited anti-nonGal IgM and IgG absent pre-transplant, and increased post-transplant with a maximum level at POD 14. (similar to anti-nonGal antibody levels in B266 and B267) (pg. 56, Fig. 3, Appendix 10). Similarly, anti-Allo IgM and IgG showed no antibody pre-transplant, and increased to a post-transplant maximum at POD 14 (pg. 57, Fig. 4, Appendix 10). Therefore, in baboon B268, the absence of an immune reaction to Gal+ skin did not result in prolongation in the survival of the GalT-KO or Allo skin grafts.

9) Baboon B269 also exhibited a similar B-cell response to baboons B266 and B267. For example, FACS data exhibited anti-nonGal IgM and IgG absent pre-transplant, and increased post-transplant with a maximum level at POD 14. (similar to anti-nonGal antibody levels in B266 and B267) (pg. 58, Fig. 1, Appendix 11). Similarly, anti-Allo IgM and IgG showed no antibody pre-transplant, and increased to a post-transplant maximum at POD 14 (pg. 59, Fig. 2, Appendix 11). Anti-Gal antibody levels peaked at POD 7 as in previous animals. Taken together, these data demonstrated that the presence of Gal+ graft does not accelerate rejection of Allo grafts.

Summary of In vitro data:

1) Primary grafting of Gal+ skin to baboons led to hyperacute rejection of the grafts (white graft), due to high levels of natural anti-Gal antibodies. Levels of anti-Gal antibodies increased after rejection and second-set grafts were again hyperacutely rejected.

2) Primary grafting of GalT-KO skin to baboons led to delayed rejection compared with Gal+ skin, and the GalT-KO skin lasted as long as allogeneic skin grafts before both rejected. The immune response to rejection of the GalT-KO was characterized by a high B cell response, as indicated by an early, specific Ab increase after rejection of the skin graft.

3) Secondary grafting of GalT-KO skin resulted in hyperacute rejection (white graft), presumably due to the high levels of anti-non-Gal antibodies produced after rejection of the first GalT-KO graft.

4) Primary grafting of Allogeneic baboon skin to baboons led to rejection in approximately the same time frame as did grafting of GalT-KO skin, but was characterized by a strong T cell response and a less vigorous B cell response, as indicated by a slower and smaller early, specific Ab increase after rejection of the skin graft. Secondary Allogeneic grafts underwent accelerated but not hyperacute rejection, also consistent with a T-cell mediated form of rejection.

KEY RESEARCH ACCOMPLISHMENTS:

- **We have shown that primate recipients of skin grafts from pigs that express the Gal antigen (Gal+) reject these grafts in a hyperacute manner, consistent with the presence of anti-Gal antibodies responsible for hyperacute rejection of pig organs transplanted to primates (baboons).** Thus, these unique, genetically engineered GalT-KO pigs will likely be required for success of pig skin xenografts.

- **We have demonstrated that primates do not exhibit this hyperacute rejection phenomenon when GalT-KO pig skin grafts are transplanted. In contrast, these xenotransplants last at least as long as primate skin allotransplants on split-thickness wounds.** This result confirms our preliminary data and has important implications for the use of GalT-KO pig skin grafts to treat battlefield injuries.

- **We have demonstrated that GalT-KO swine skin can cover full-thickness wound beds (analogous to those expected in battlefield wounds) in primates equally as well as allogenieic skin grafts.** Previous studies used split thickness wound beds. Data from full-thickness wound beds are comparable to split- thickness data. Histologically, the full-thickness bed is a better model, as analysis of these wounds can be performed without the confounding artifact of migration of peripheral skin cells into the wound area during healing. In addition, full-thickness wounds better represent the clinical situation, where 3rd and 4th degree burns require immediate treatment.

- **We have demonstrated no appreciable difference in graft function between fresh vs. frozen and thawed skin grafts from either swine or baboon sources.** Thus frozen/thawed skin lasts at least as long as fresh skin.

- **We have elucidated possible differential immunologic mechanisms involved in the response to xeno vs allo skin grafts following first or second transplants:**
    - Both Gal KO and Gal+ skin regrafting in sensitized animals led to high B cell responses, as indicated by early, specific Ab increases, resulting in hyperacute rejection (white graft).
    - Allo skin regrafting in sensitized animals was followed by higher T cell responses and lower B cell responses than GalT-KO regrafting, as indicated by markedly increased T cell responsiveness without a correspondingly high, early Ab increase. The increased T cell responsiveness was likely responsible for accelerated rejection, but not a white graft.

REPORTABLE OUTCOMES:

Reportable outcomes that have resulted from this research include:

- Manuscripts- "Prolonged Survival of GalT-KO Swine Skin on Baboons"- accepted by the journal *Xenotransplantation* (manuscript appears in Appendix 13).

- Abstracts- "Gal-KO Xenoskin Graft Survival is Comparable to Skin Allotransplantation for Burn Injury", accepted to Plastic Surgery Research Council, San Francisco, CA, May, 2010 (Abstract appears in Appendix 14).

- Presentations- Transplantation Biology Research Center Presentation (Presentation in Appendix 15).

- Patents and licenses applied for and/or issued – Covered by our GalT-KO patents

- Degrees obtained that are supported by this award- None

- Development of cell lines- None

- Tissue or serum repositories- None

- Informatics such as databases and animal models, etc.- None

- Funding applied for based on work supported by this award- Angelo Leto Barone, M.D.- Harvard University School of Medicine Tosteson Fellowship, 2010-2011.

- Employment or research opportunities applied for and/or received based on experience/training supported by this award:

- Angelo Leto Barone, M.D.- Research Fellow , Transplantation Biol ogy Research Center, Massachusetts General Hospital/Harvard Medical School;
- Radbeh Torabi, M.D.- Research Fellow, Tr ansplantation Biology Research Center, Massachusetts General Hospital/Harvard Medical School.

CONCLUSION:

Summary of Results:

The **importance and implications of the completed research** in this tim e period include: 1) confirm ation that our genetically-modified pigskin grafts should perform as well as human cadaveric allogeneic skin grafts as a temporary biologic cover for severe burn injuries; 2) demonstration that ***genetically-modified GalT-KO skin grafts function well after freezing and thawing;*** 3) demonstration that ***genetically-modified GalT-KO skin grafts*** provide an ***effective cover for the full-thickness skin injuries*** tha t ar e typica lly s een in burn injuries sustained in combat/ 4) demonstration that a short course of ***immunosuppression may enhance the duration and quality*** of these grafts; and 5) *in vitro* findings suggesting that antibody responses are likely to be more prevalent in the rejection of GalT-KO skin than in the rejection of allogeneic skin – suggesting that targeting of the B cell response to non-Gal antigens may improve results of GalT-KO skin grafts.

"So What" Section:

Evaluation of the results obtained during the first 8 months of this project have provided proof of concept for GalT-KO xenogeneic skin grafts under a num ber of different experim ental circumstances. Based on these results, we can envision a frozen, readily available m ilitary burn dressing, capable of being transported in a m edic's pack, th at could be used as a lifesaving tem porary skin graft for immediate, sterile coverage of critically-injured areas of a blast-wounded or flame-burned soldier's body. The ability to quickly cover such wounds would prev ent life-threatening infection and fluid/electrolyte loss while the com batant is evacuated to tertiary -care centers for definitive trea tment [1-6]. The Gal-KO pig skin graft would provide wound coverage for as m any as 7-10 days post-injury before requiring definiti ve replacem ent with a permanent autograft. The treatm ent approach would replace or provide an adjunct to the current m ethod of utilizing hu man cadaver allog raft s kin as a temporary dressing- an extrem ely effectiv e techn ique that is underutilized due to a lack of availa bility, portability, cost-effectiveness, as well as ethical and infectious disease concerns associated with the use of human tissue.

In addition, previous studies of responses to allogene ic vs. xenogeneic transplant s [12] m ake it likely that neither GalT-KO nor allogeneic sk in grafts will sensit ize for each oth er, so that s equential grafts m ay be possible, thereby extending total surv ival of the tem porary cover for over two weeks. A herd of a ppropriate skin graft donor anim als could be m aintained for this purpose and could provide an attractive alternative to human cadaveric allograft skin as an em ergency tem porary graft. S ince hum an cadaveric allogeneic skin grafts are the current "gold standard" for temporary skin grafts [6-10], and since our genetically-modified pig skin would have significant advantages related to ava ilability, cost, safety and ethical considerations, we intend to develop model further as a new approach to the initial treatment of severe battlefield injuries.

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APPENDIX 1

PRELIMINARY DATA

Results of a pig-to-primate skin graft using a GalT-KO pig: In an attempt to evaluate whether the use of skin from GalT-KO swine would be of benefit in prolonging the survival of pig-to-primate skin grafts, we performed one proof-of-principle experiment. The recipient was B234, a 3-4 year old baboon that had previously been used in another experiment 8 months earlier, involving thymectomy and treatment with an immunosuppressant. By the time of this skin experiment, the baboon was healthy, and its blood cell counts and phenotypes had returned to baseline.

On the day of skin transplantation, a partial thickness section of skin (approximately 2x8 inches) was taken from the right dorsal shoulder horizontally towards the spine using a dermatome set at a depth of 24mm. Onto this skin bed, we placed four skin grafts side-by-side, as follows (left to right): 1) self skin, 2) GalT-KO pig skin from pig 16006, 3) Gal positive pig skin from pig 17944; and 4) skin from another baboon (allograft) B199 (Fig. 5A). The Gal-positive skin was from a pig genetically matched to the GalT-KO pig (except for the fact that the Gal-positive animal did contain the α-1,3-galactosyltransferase gene). These skin grafts were obtained from the respective donors using the same dermatome with the same settings, although the blade was fresh for each animal. The skin grafts were kept moist and cold in a saline-filled Petri dish on ice while being trimmed to the proper size to fit the graft bed. The grafts were then sutured into place using 2-0 Ethilon. Biopsies for frozen and formalin samples were taken from the discarded portions of each graft. The wound was then covered with Bacitracin and gauze, and a jacket was placed to prevent the baboon from scratching the wound.

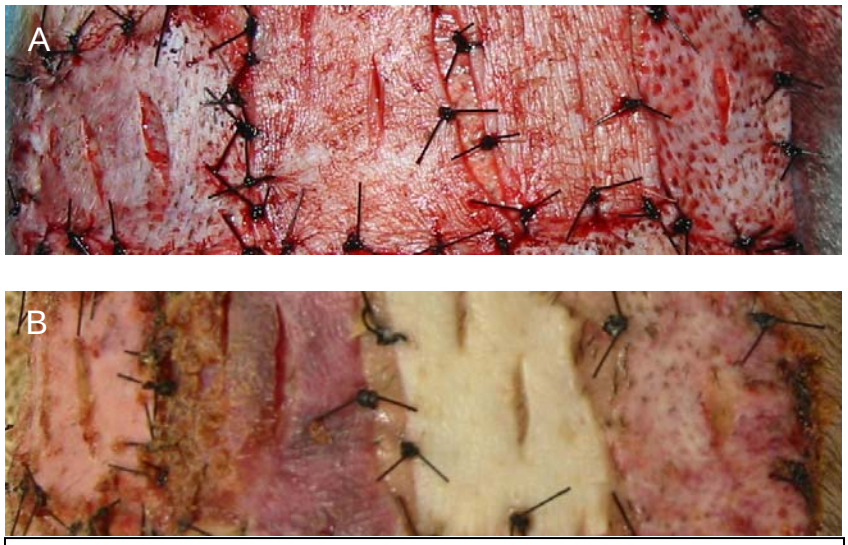


Figure 5: Skin grafts (from left to right): self; GalT-KO; Gal positive; allogeneic baboon. A) day 0; B) day 4 post-grafting

Clinical course and gross findings: The animal was sedated and anesthetized to evaluate the skin grafts and draw blood on postoperative days 4, 7, 11, 15, 21, and 30. On each of these days, we examined, cleaned, and photographed the wound, took frozen and formalin biopsies, drew blood for CBC and immunologic assays, and redressed the wound. By day 4, the

self and allo grafts were warm, pink, and healthy, implying that they had engrafted and begun to re-vascularize (Fig. 5B). The GalT-KO skin was also warm and pink, although demonstrating a slightly mottled appearance, indicating that it too had vascularized. In stark contrast, the Gal positive pig skin was cool and bright white, although still intact, indicating a "white graft", as previously described for skin grafts that are rejected hyperacutely due to preformed antibodies [50;51]. We inferred that this white graft had never engrafted nor re-vascularized (Fig. 5B), indicating a clear difference in behavior from the GalT-KO skin graft even as early as four days.

By day 7, the self skin was still pink and healthy, but the allo skin had begun to show rejection, as indicated by a brown, scab-like crust forming over the graft, similar to the appearance of the Gal positive pig skin at that time. In contrast, the GalT-KO skin was still warm, dark pink, and mostly intact, although a local superficial infection had caused some loss of integrity. This infection cleared after 3 days of 15 mg/kg vancomycin iv, and both the apparent inflammation and elevated white count returned to normal. By day 9, the allo skin had been totally rejected and was covered by scab, but a small amount of GalT-KO skin still remained intact. The self skin still appeared normal.

In summary, these gross examinations revealed that the Gal positive skin never engrafted, while allogeneic skin engrafted but was rejected by day 8-9. The GalT-KO skin appeared to have lasted even longer than the allograft, becoming vascularized in a similar fashion and being rejected only by day 11. These preliminary data suggest that GalT-KO skin may serve as well or better than allogeneic skin as an acute live skin covering for severe burn injuries.

Histologic examination: Histology of the skin grafts on animal B234 are shown in Fig. 6. Histology of the self skin showed normal skin except for a small amount of non-specific granulation tissue on day 7 and evidence of localized bacterial infection on day 9, consistent with gross inspection (above). Also consistent with gross observations, histology of the allo skin appeared to be normal on days 0 and only slightly vacuolated on day 4, but developed a cellular infiltrate consistent with rejection by day 7. By day 9, the allo skin was rejected and the pathology revealed only the underlying regenerating host skin bed.

Consistent with the gross observation of a white graft, the normal pig (i.e. Gal positive) skin never showed histologic evidence of engraftment. Histology on day 4 showed thrombi in small vessels, consistent with hyperacute rejection, leading to occlusion of the blood vessels, presumably due to preformed anti-Gal antibody. By day 7, the graft was necrotic (Fig. 6). In contrast, histologic examination of the GalT-KO skin graft showed engraftment of relatively normal skin on days 4 and 7, with only mild congestion. By day 9, the graft showed evidence for incipient cellular rejection similar to that seen in the allograft by day 7.

In summary, the histologic findings supported those from gross examination, and indicated that GalT-KO skin engrafted and re-vascularized in a baboon as well as or better than allogeneic skin, while normal pig skin was rejected hyperacutely. Again,

these results support the hypothesis that GalT-KO skin may serve as well or better than allogeneic skin as an acute live skin covering for severe burn injuries.

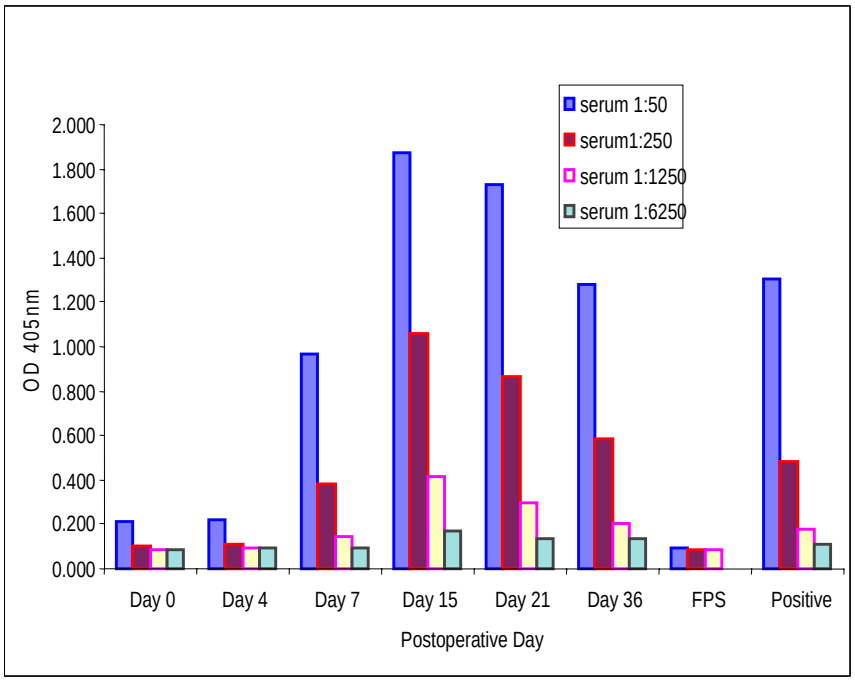


Figure 7: Sequential anti-Gal IgG ELISA assays following skin grafts on B234

In vitro analyses of anti-Gal antibodies: The results of an anti-Gal ELISA assay on sequential serum samples from baboon B234 are shown in Fig. 7. As seen in this figure, there were already low baseline levels of preformed anti-Gal IgG in this baboon at days 0 and 4. The levels started to rise by day 7 and peaked by day 15, indicating that the baboon had been further sensitized to the Gal antigen by exposure to the Gal positive skin graft. Levels of preformed anti-Gal IgM (not shown) were higher initially and also increased greatly after day 4, peaking at day 15. Additional experiments showed that the preformed anti-Gal IgM was largely

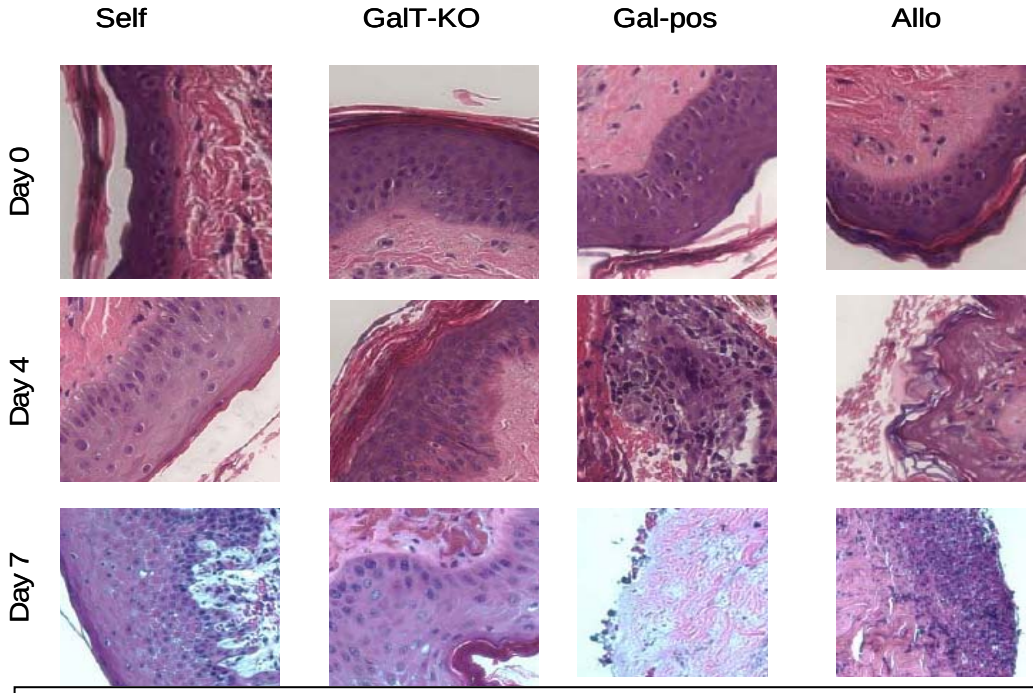
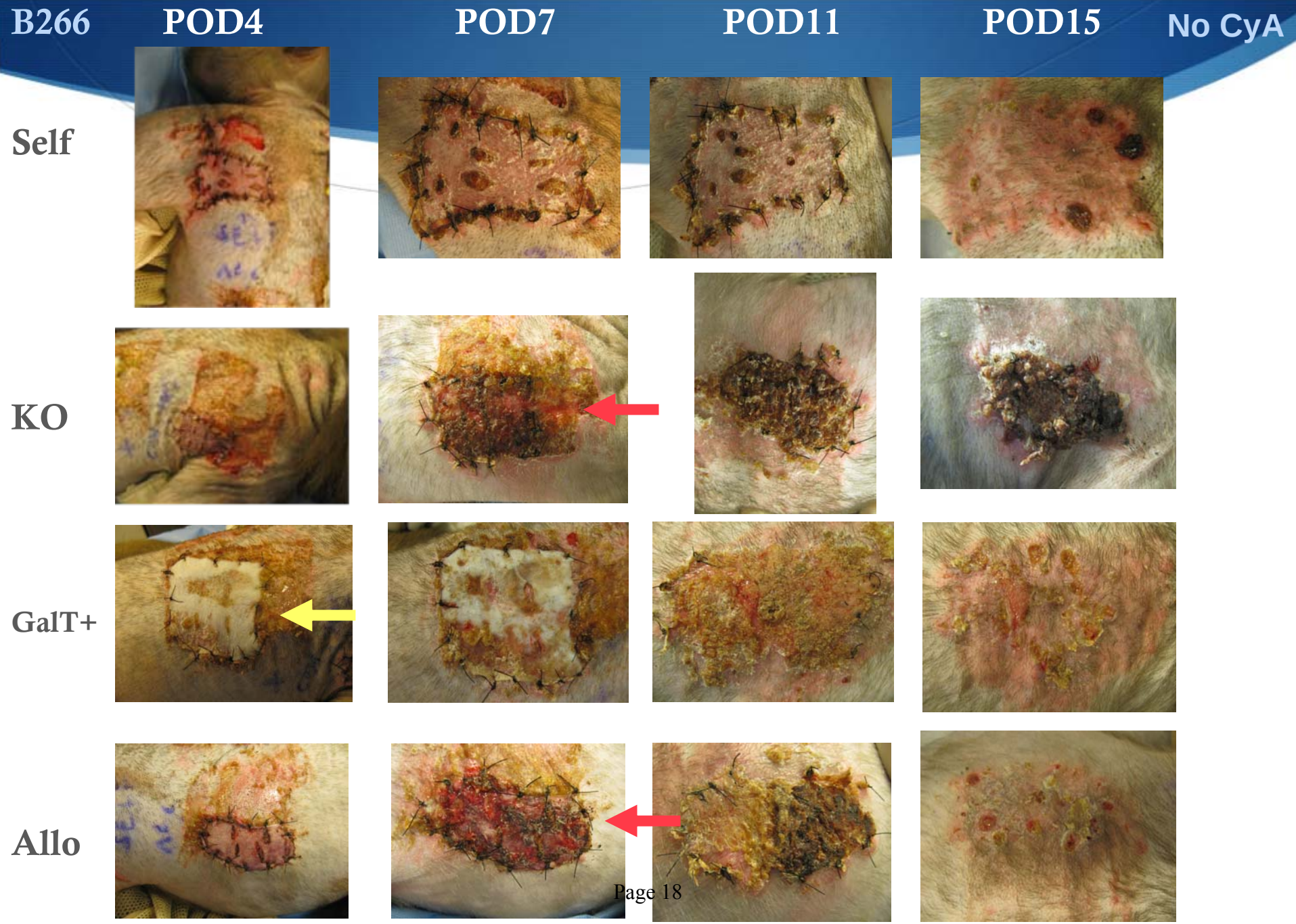


Figure 6: Histology of skin grafts on baboon B234, biopsies on days 0, 4 and 7

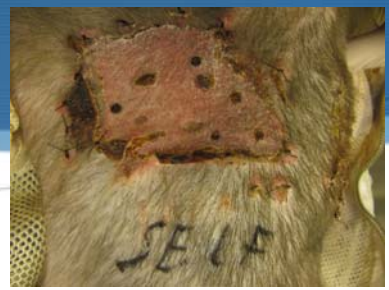
responsible for the cytotoxicity against Gal positive tissue.


XENO SKIN GRAFTS
B266
No CyA IM
B266
SELF
18811
GalT +
18671
GalT- KO
B267
Allo
B267
CyA IM
B267
SELF
18811
GalT +
18671
GalT- KO
B266
Allo

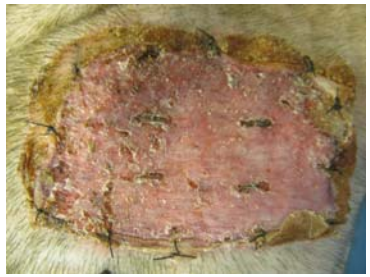

B266
POD4
POD7
POD11
POD15
No CyA
Self
KO
GalT+
Allo

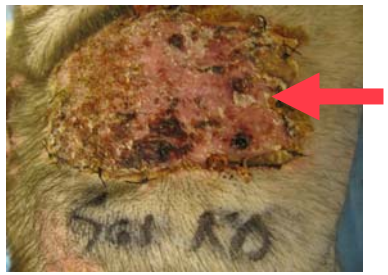
B267 POD4 POD7 POD11 POD15 CyA

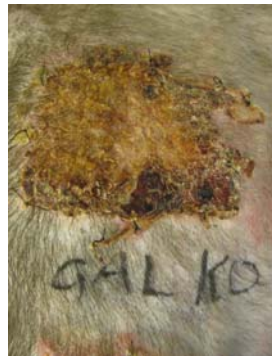
Self

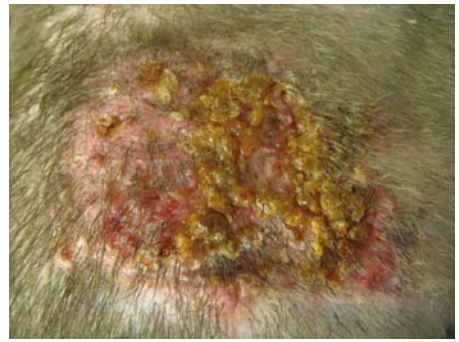


KO

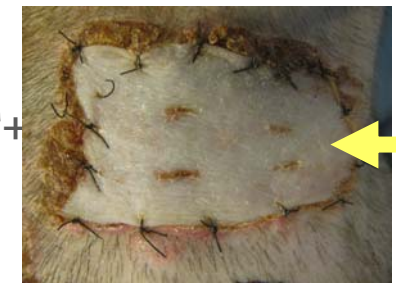


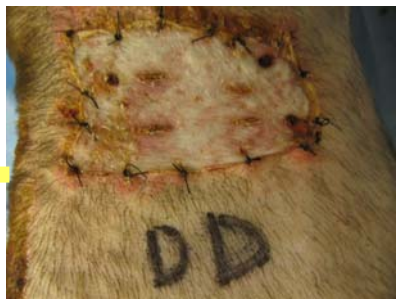


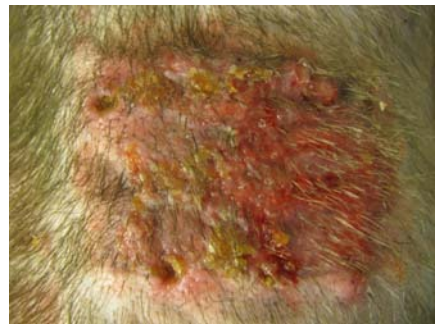




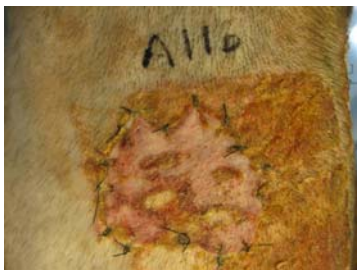
GalT+

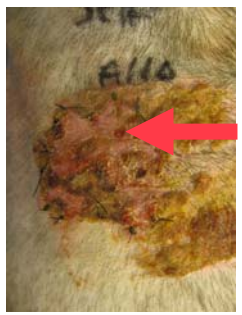


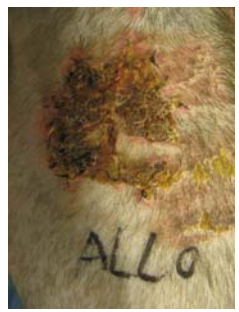


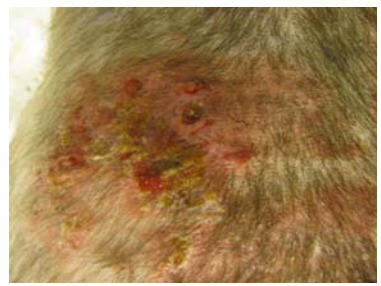


Allo

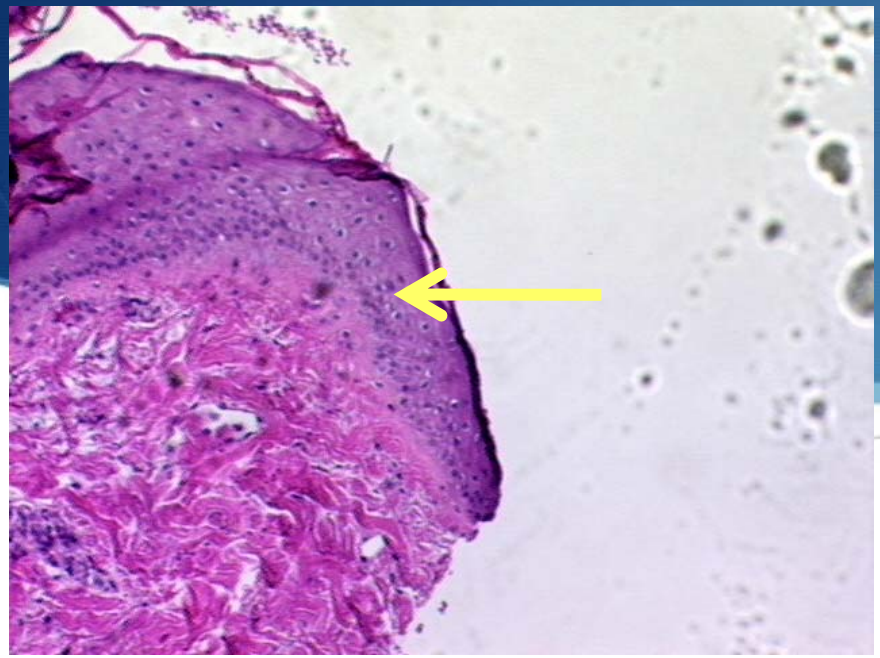




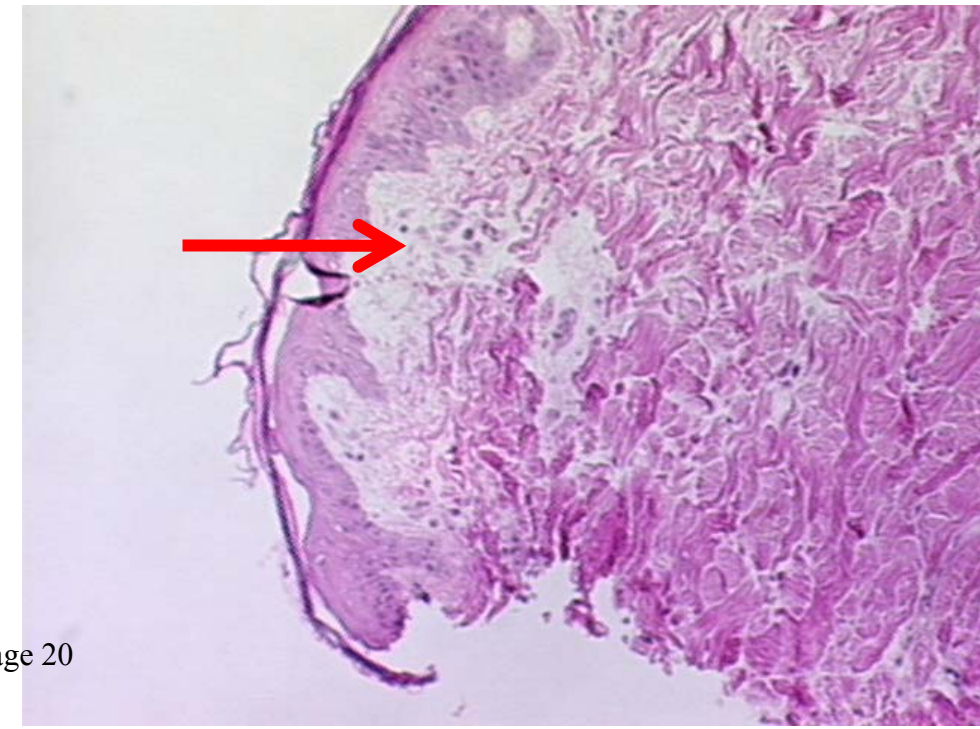


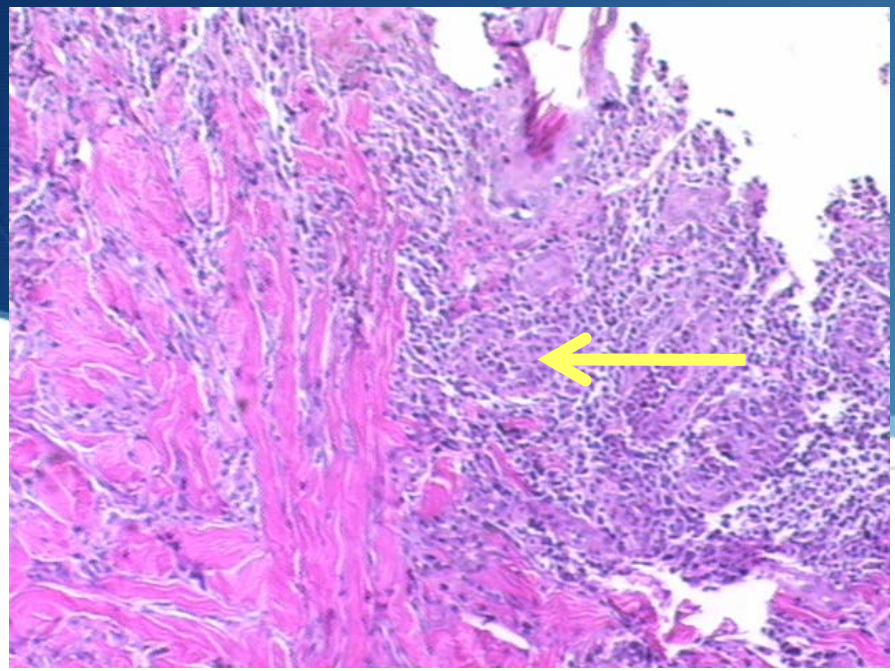


Self skin POD4



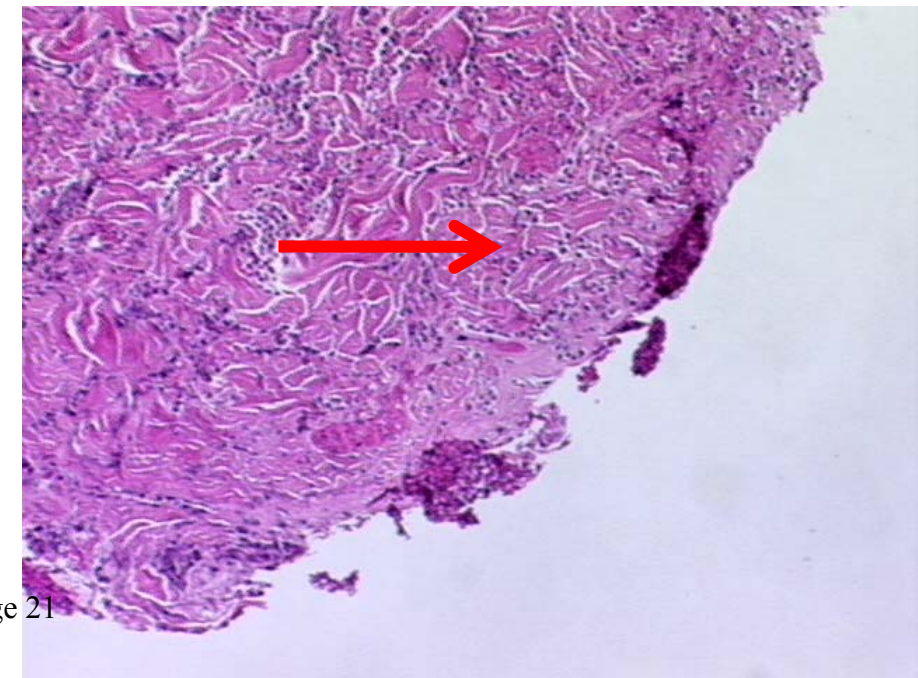
GalT+ POD4

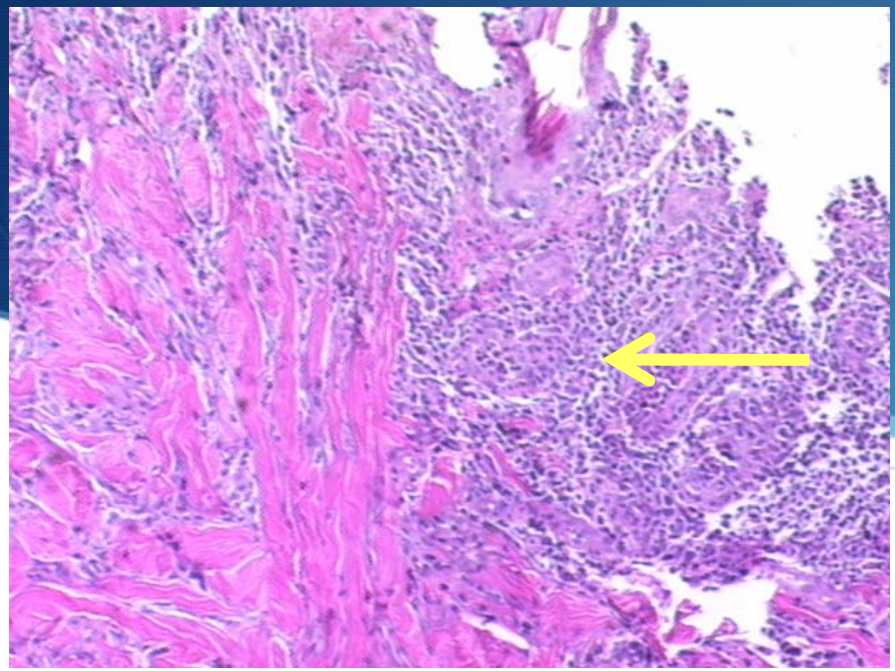




B266 GalT-KO POD7 (no CyA)

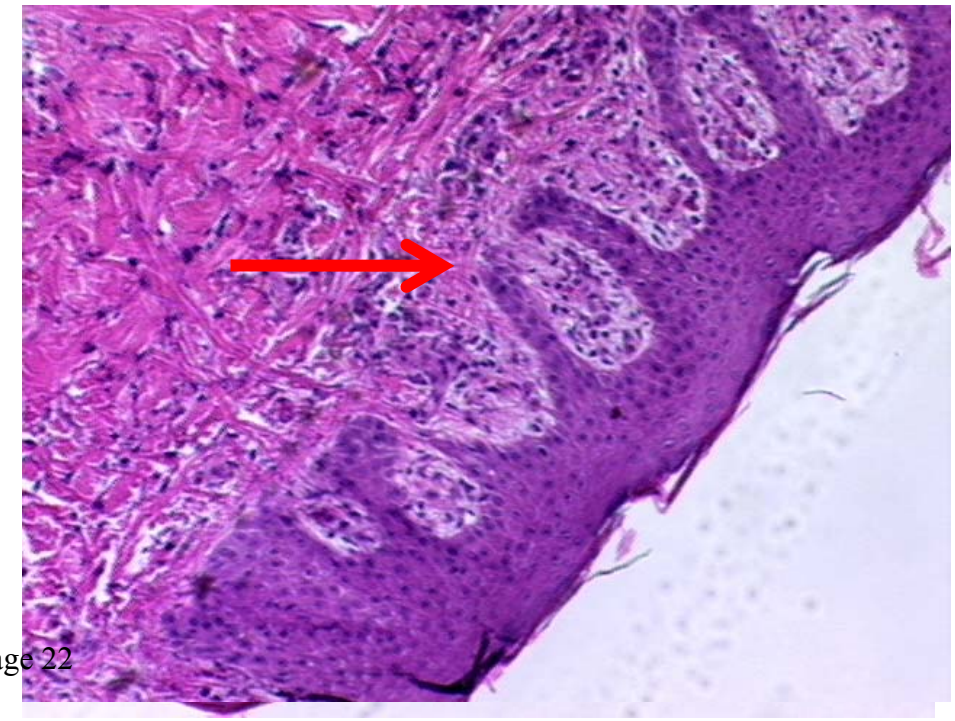
B266 Allo POD7 (no CyA)



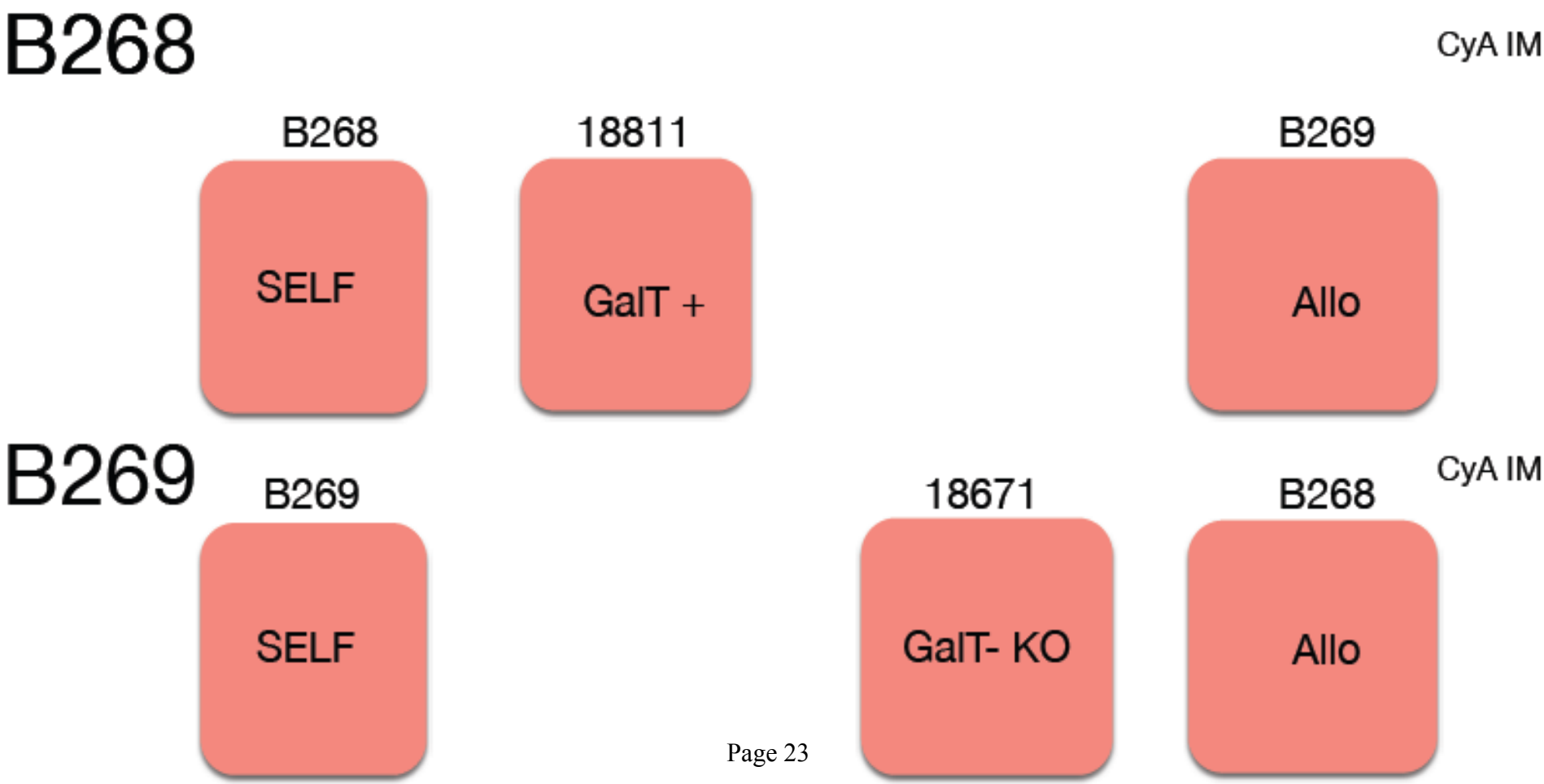


B266 GalT-KO POD7 (no CyA)

B267 GalT-KO POD7 (CyA)

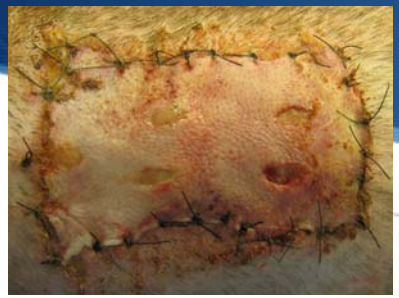
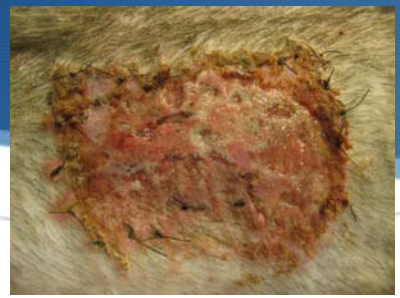
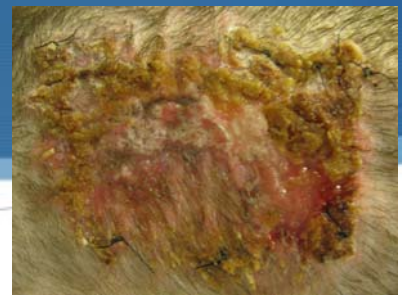
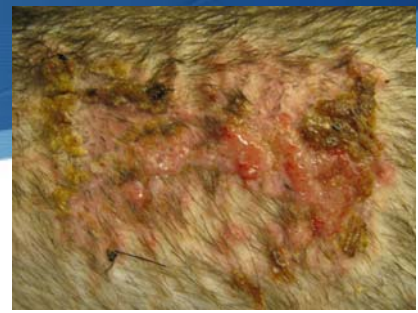


XENO SKIN GRAFTS

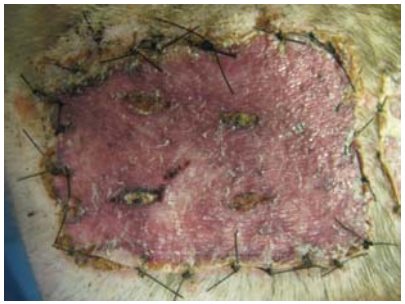
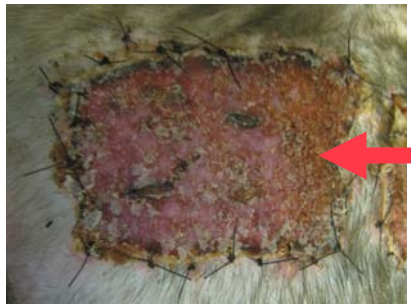
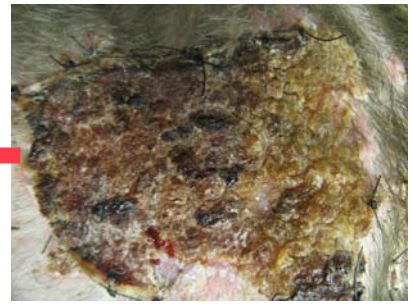
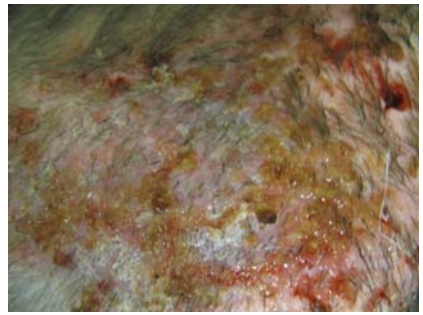


B268 POD4 POD7 POD11 POD15 CyA

Self

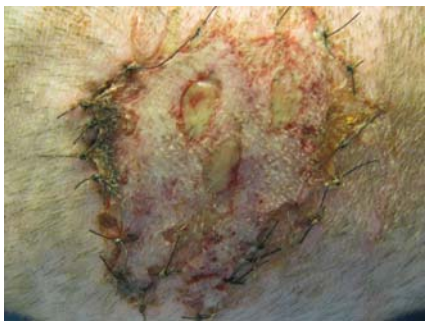
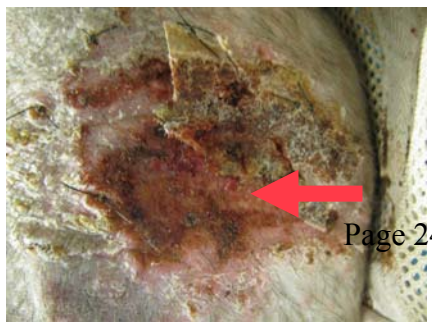
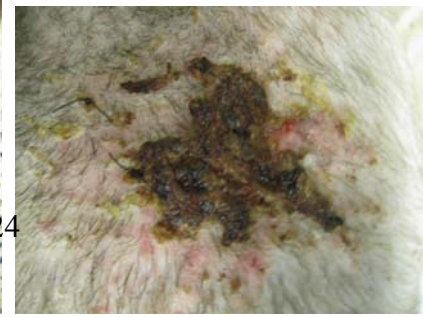
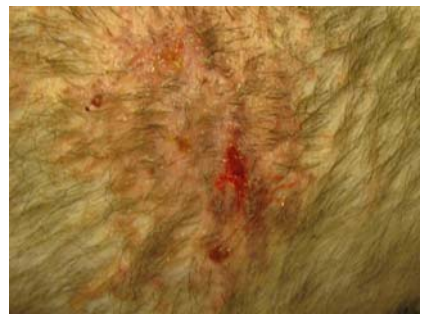





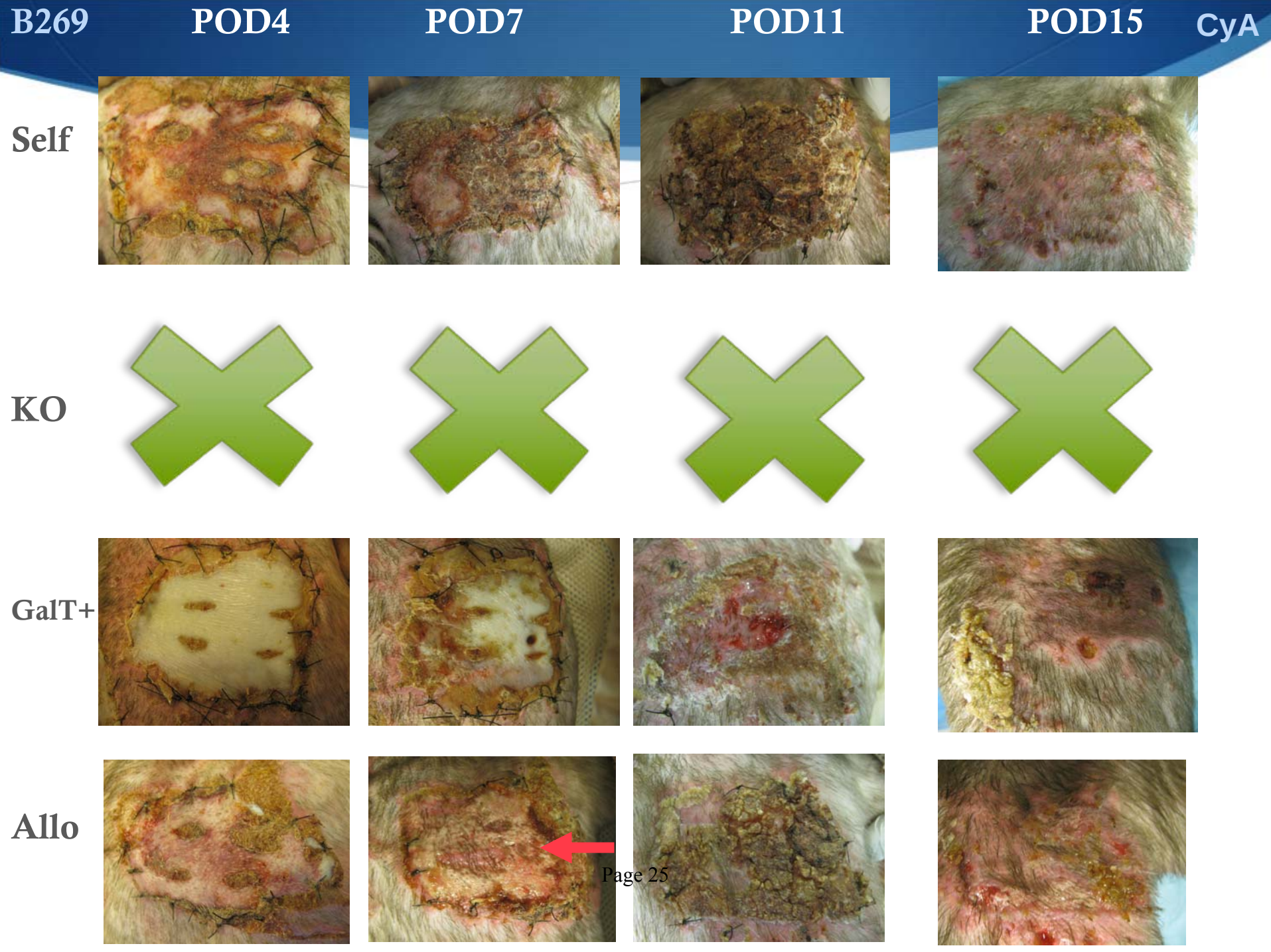
KO

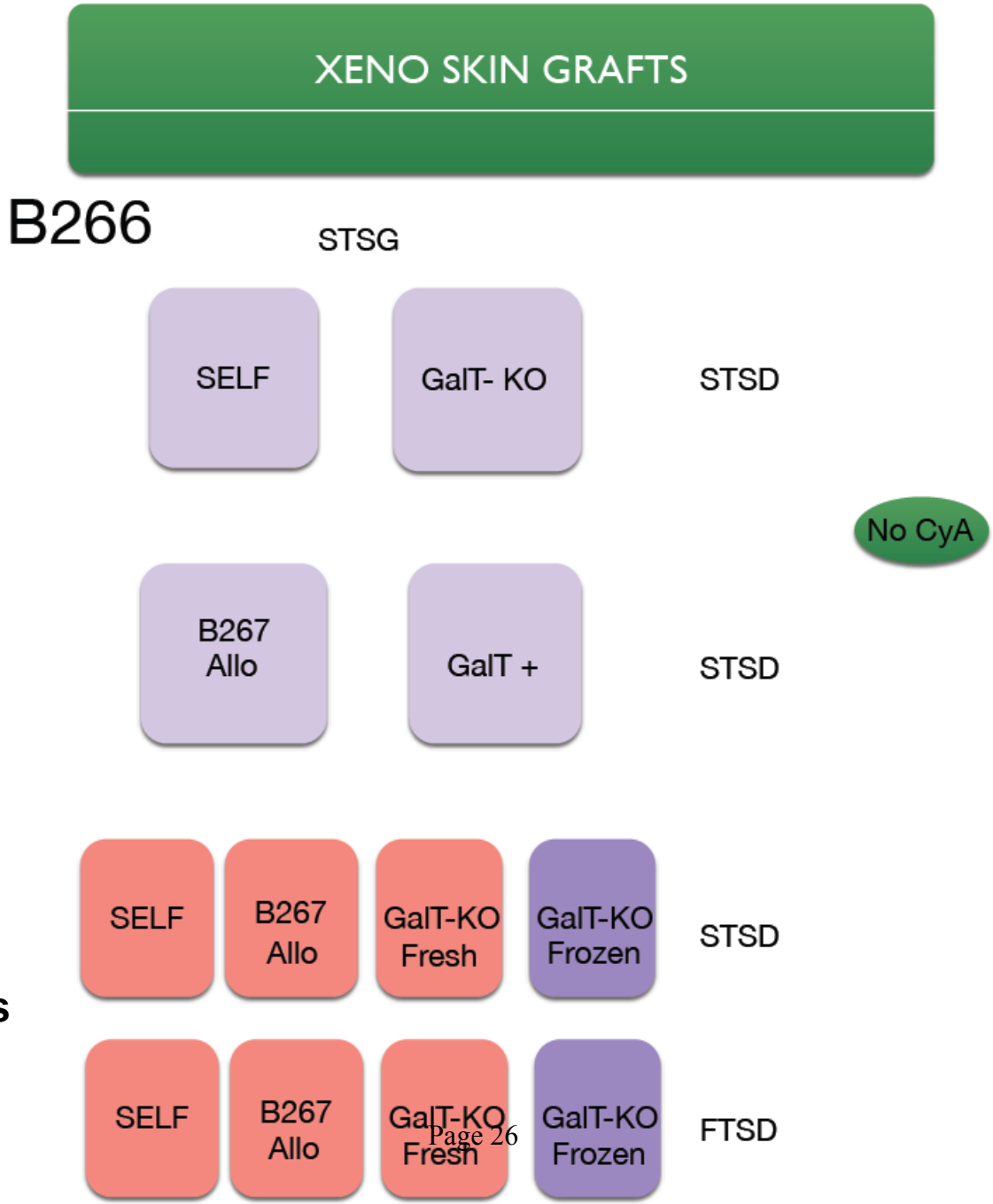





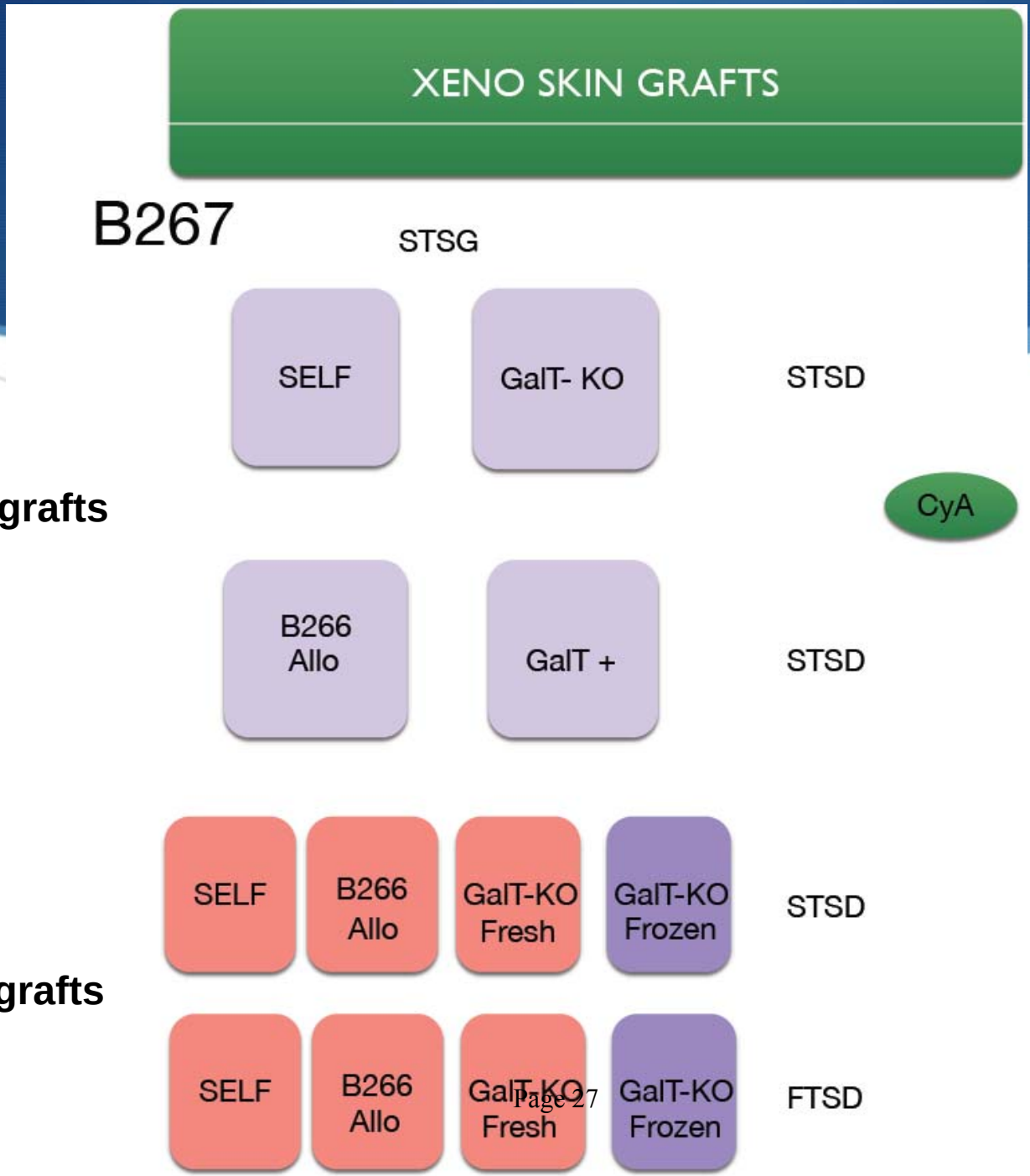
GalT+

Allo


B269
POD4
POD7
POD11
POD15
CyA
Self
KO
GalT+
Allo


XENO SKIN GRAFTS
B266
STSG
SELF
GalT- KO
STSD
No CyA
1st set of grafts
B267 Allo
GalT +
STSD
SELF
B267 Allo
GalT-KO Fresh
GalT-KO Frozen
STSD
2nd set of grafts
SELF
B267 Allo
GalT-KO Fresh
GalT-KO Frozen
FTSD

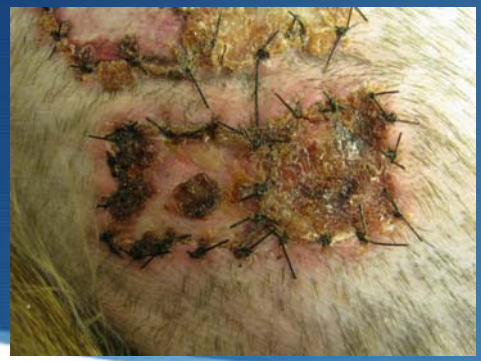


1st set of grafts

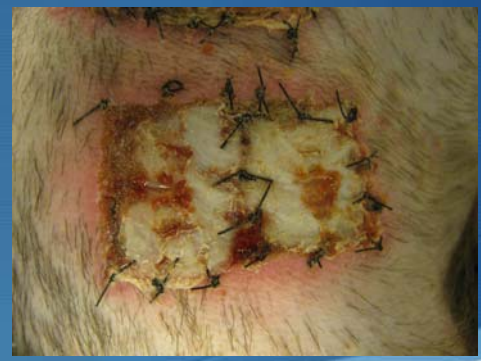
2nd set of grafts

Full Thickness

POD4

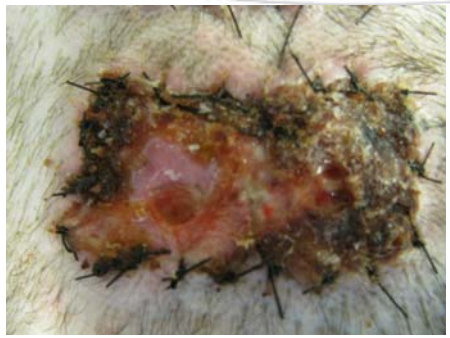


Self Allo

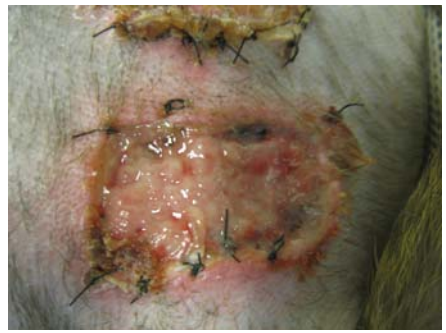


KO fresh KO frozen

POD7

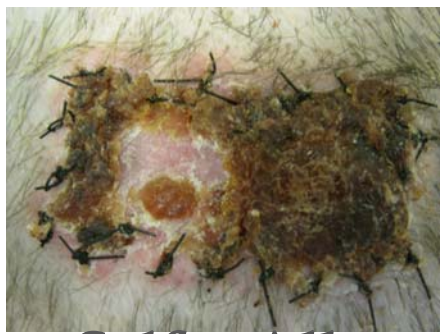


Self Allo

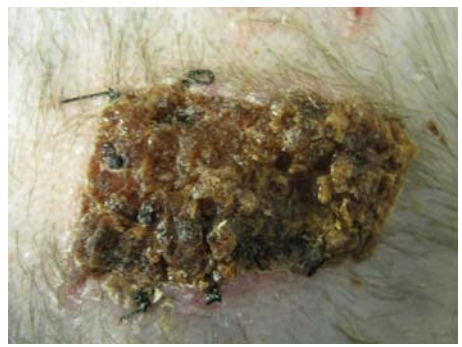


KO fresh KO frozen

POD 10



Self Allo

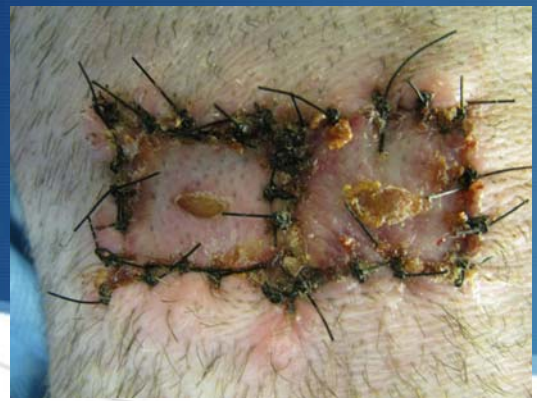


KO fresh KO frozen

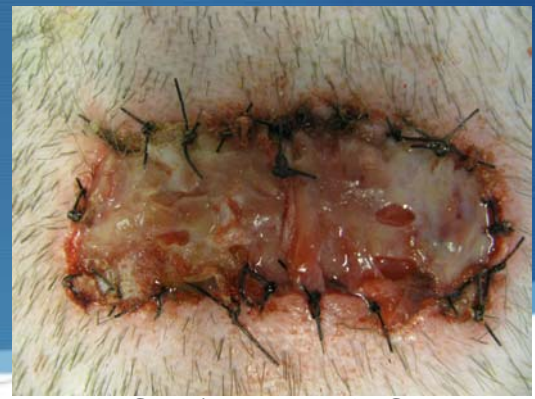
No CyA

Full Thickness

POD4

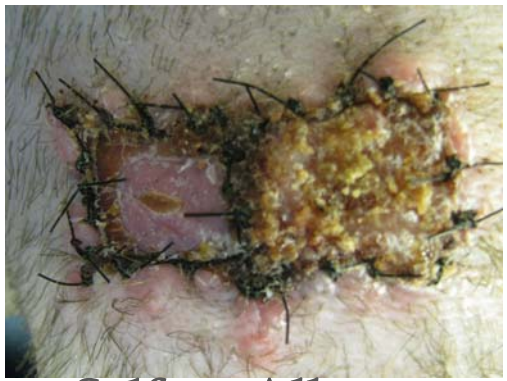


Self Allo

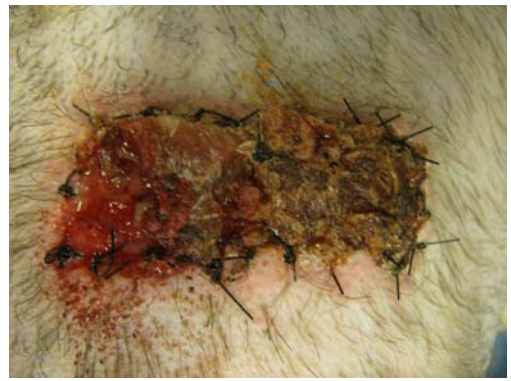


KO fresh KO frozen

POD7

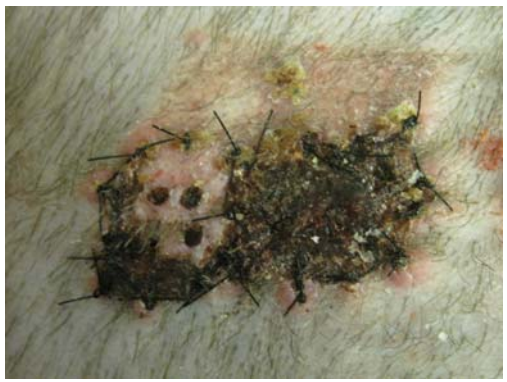


Self Allo

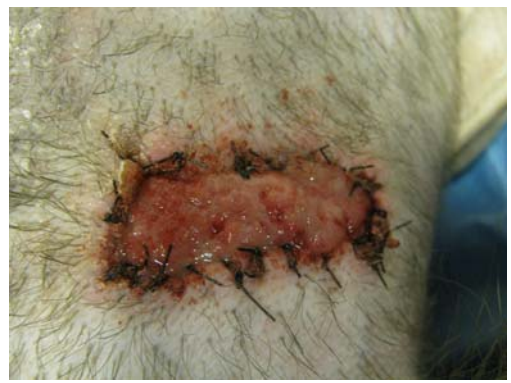


KO fresh KO frozen

POD 10

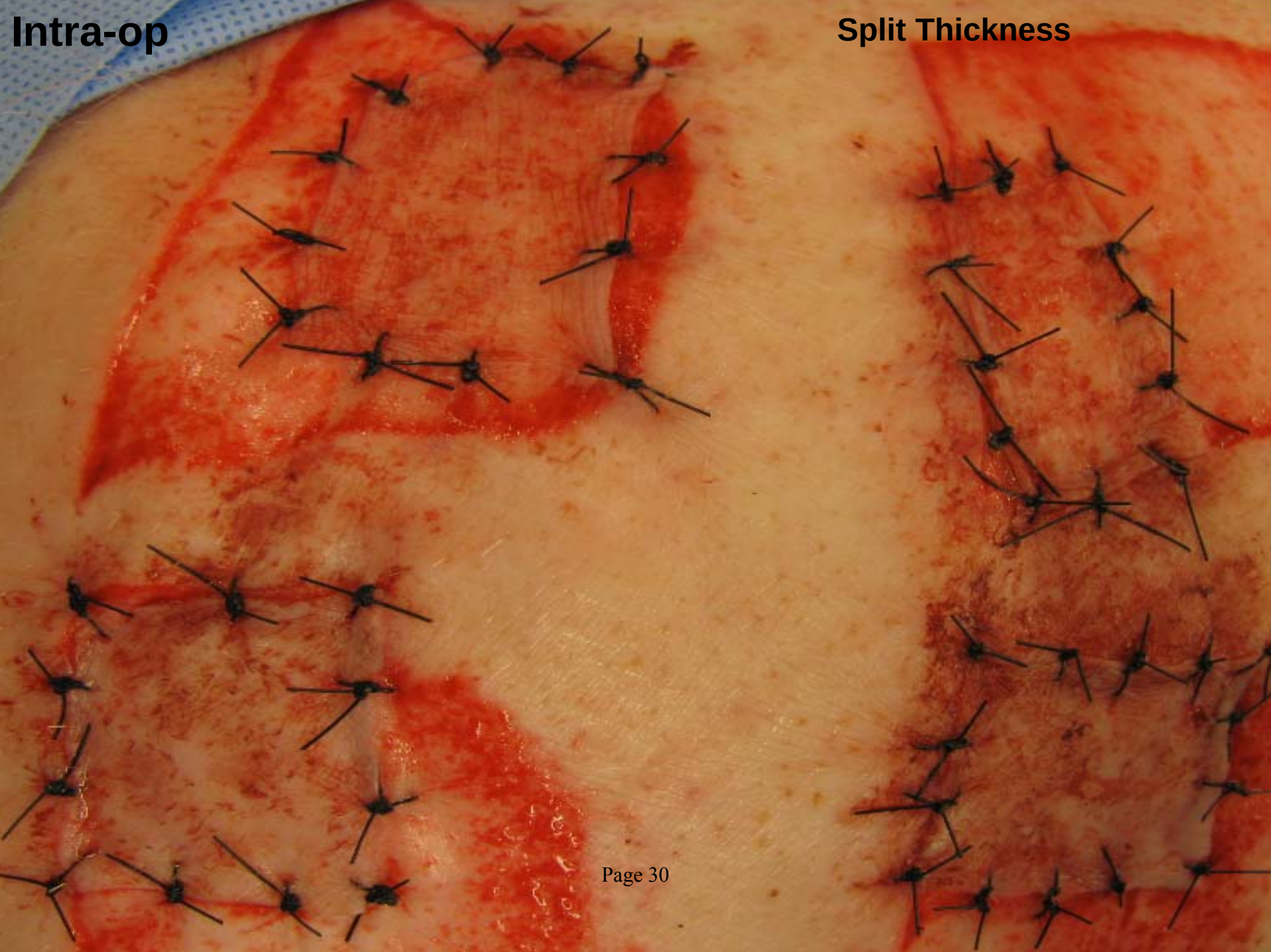


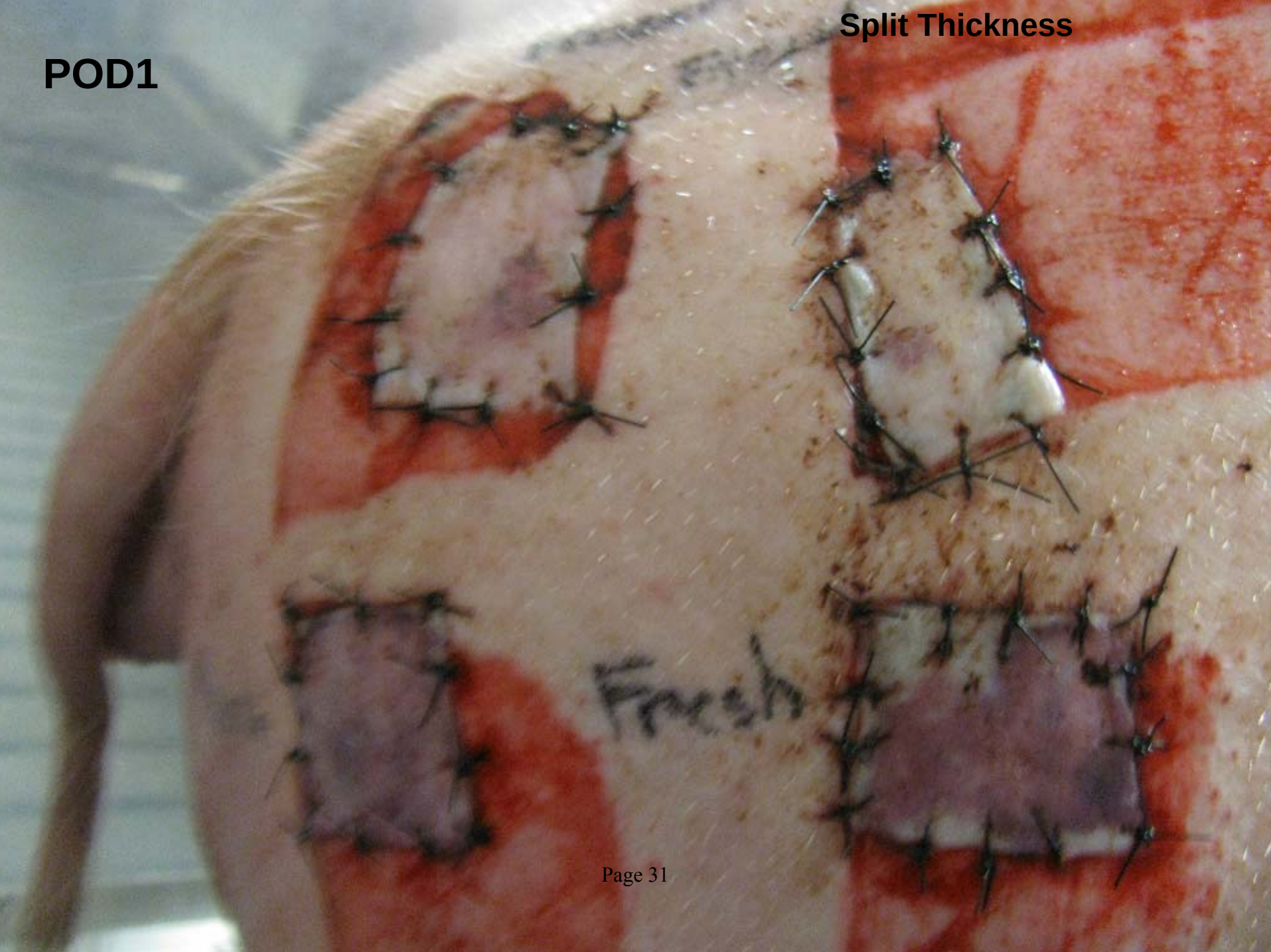
Self Allo

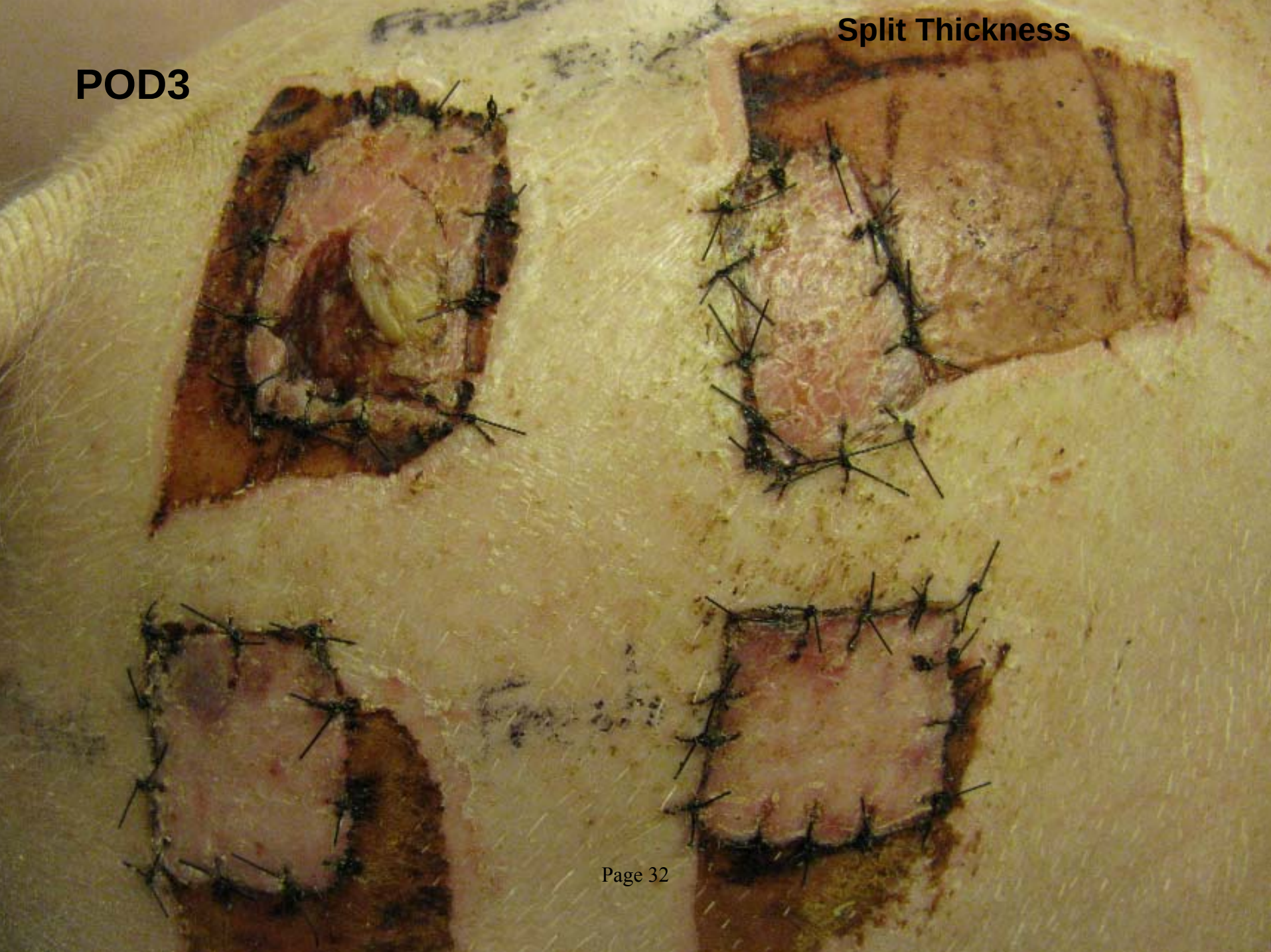


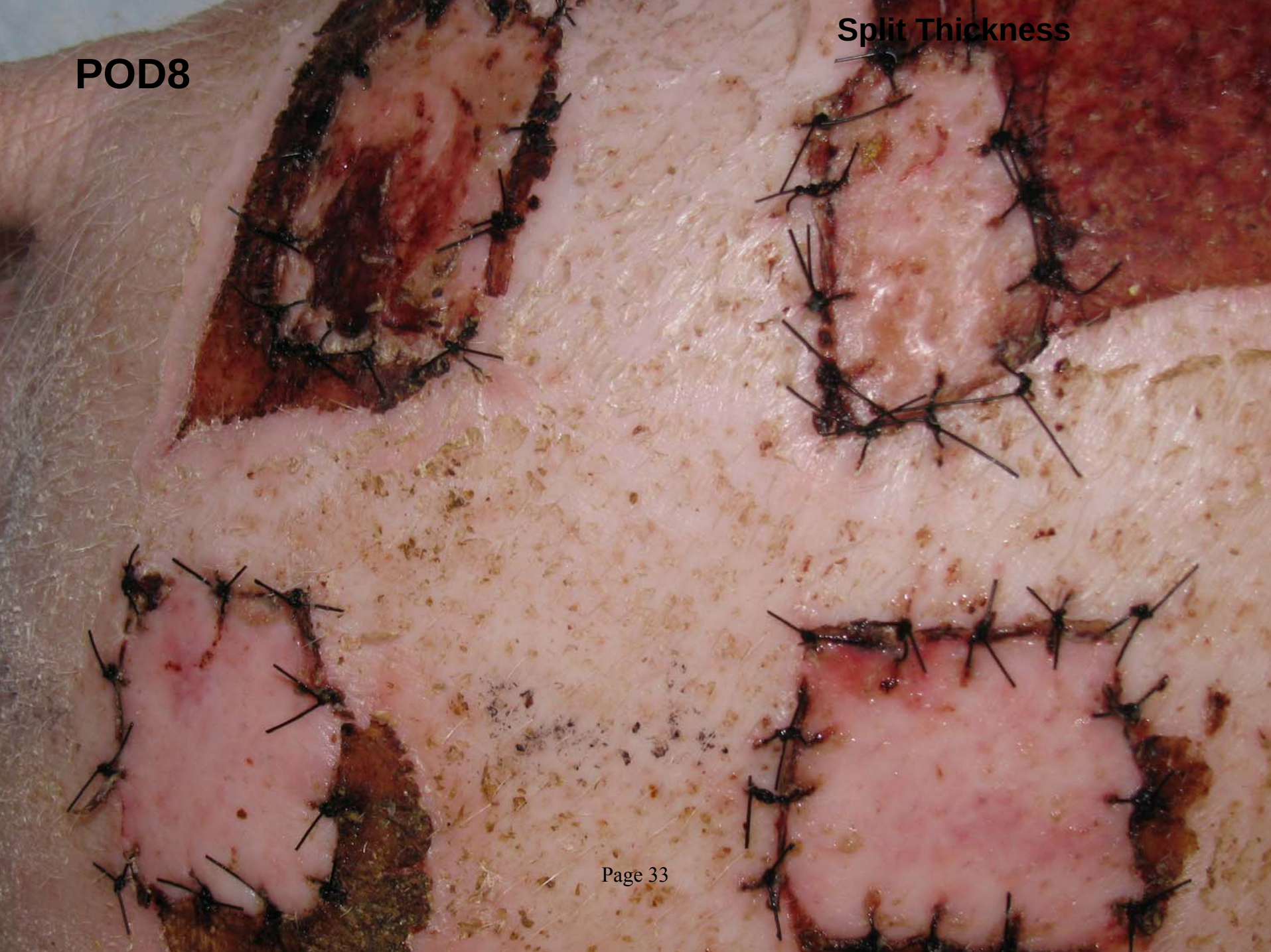
KO fresh KO frozen

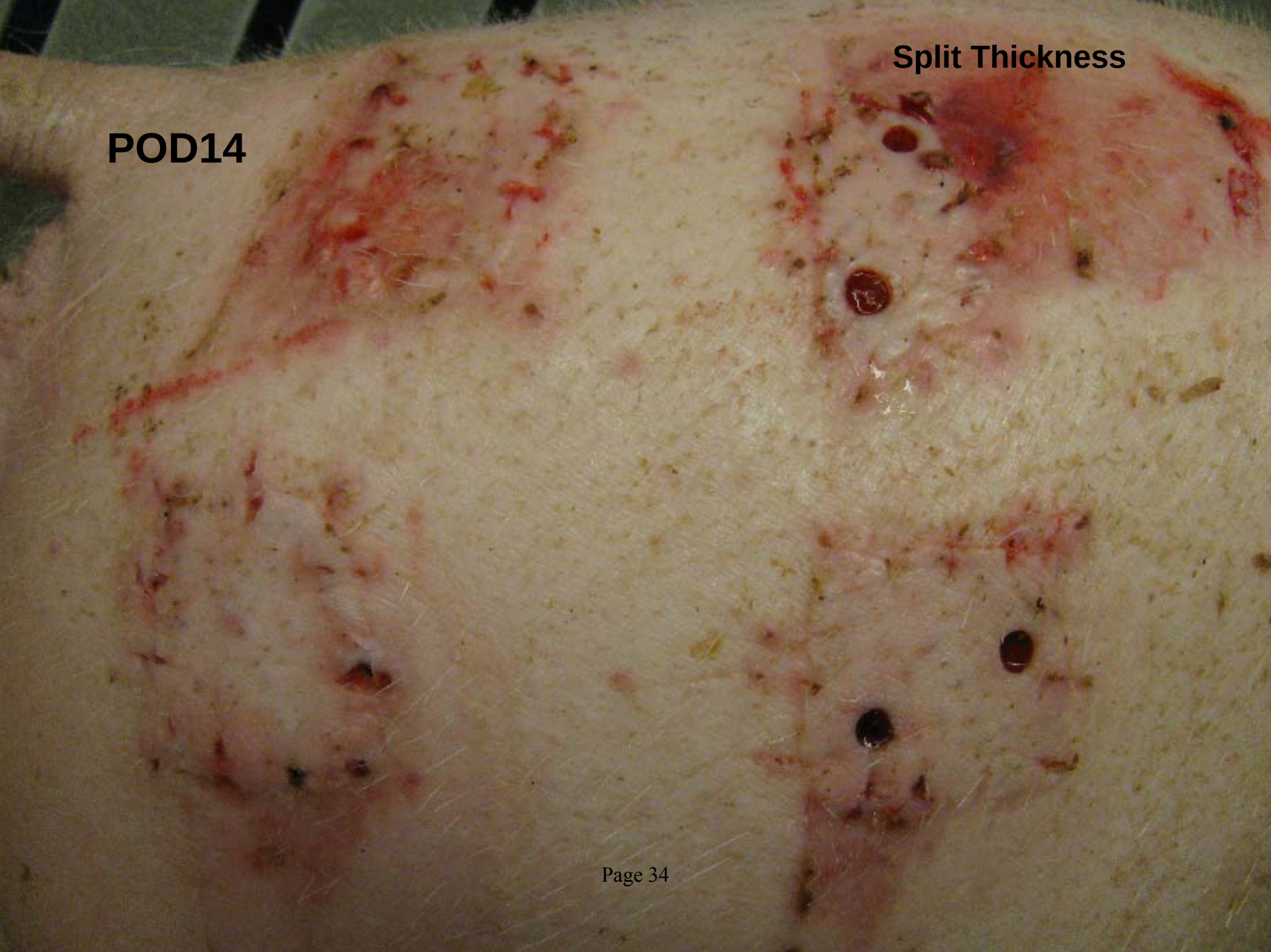
CyA


Intra-op
Split Thickness

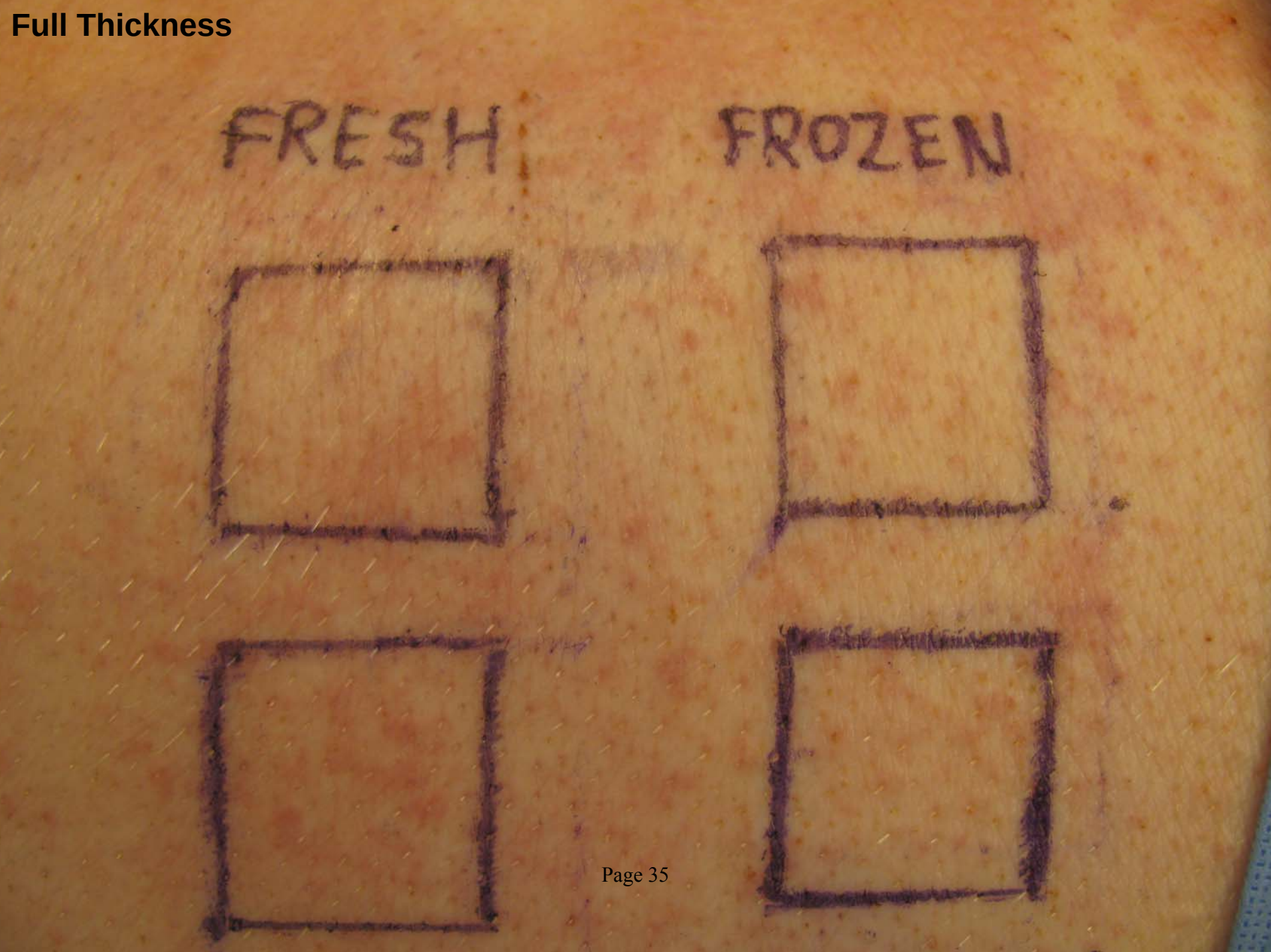

Split Thickness
POD1


POD3
Split Thickness

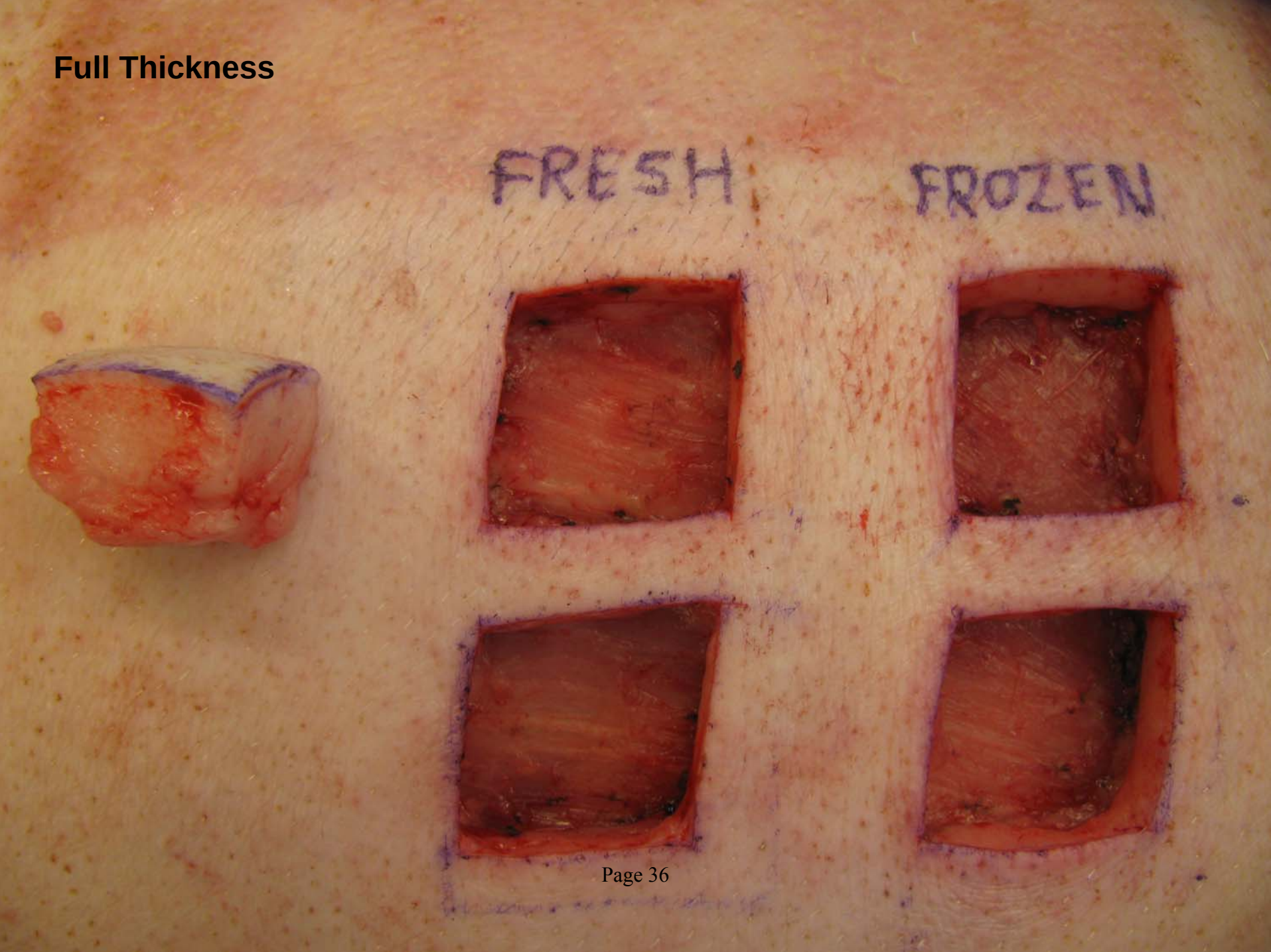

POD8
Split Thickness

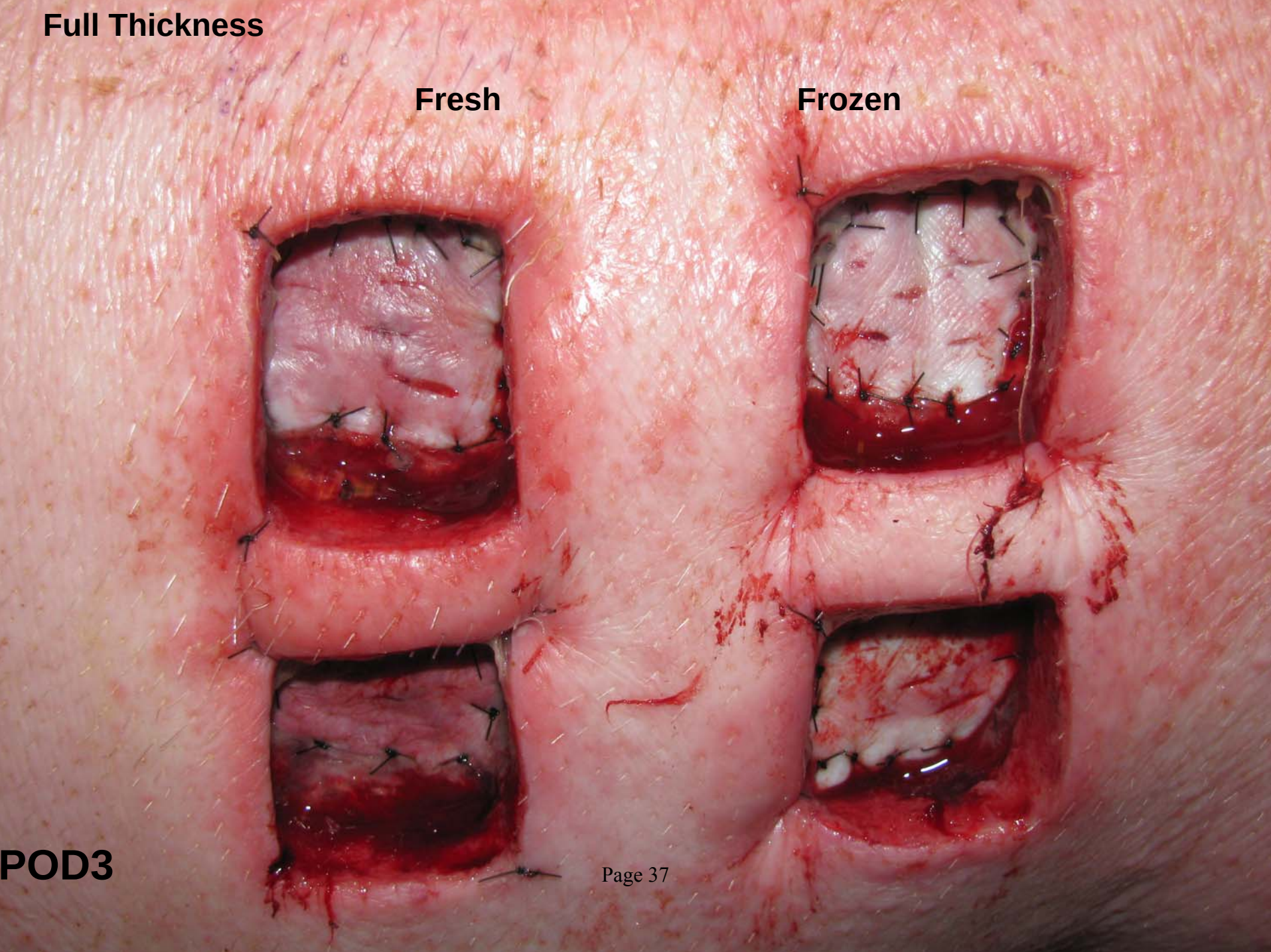

Split Thickness
POD14

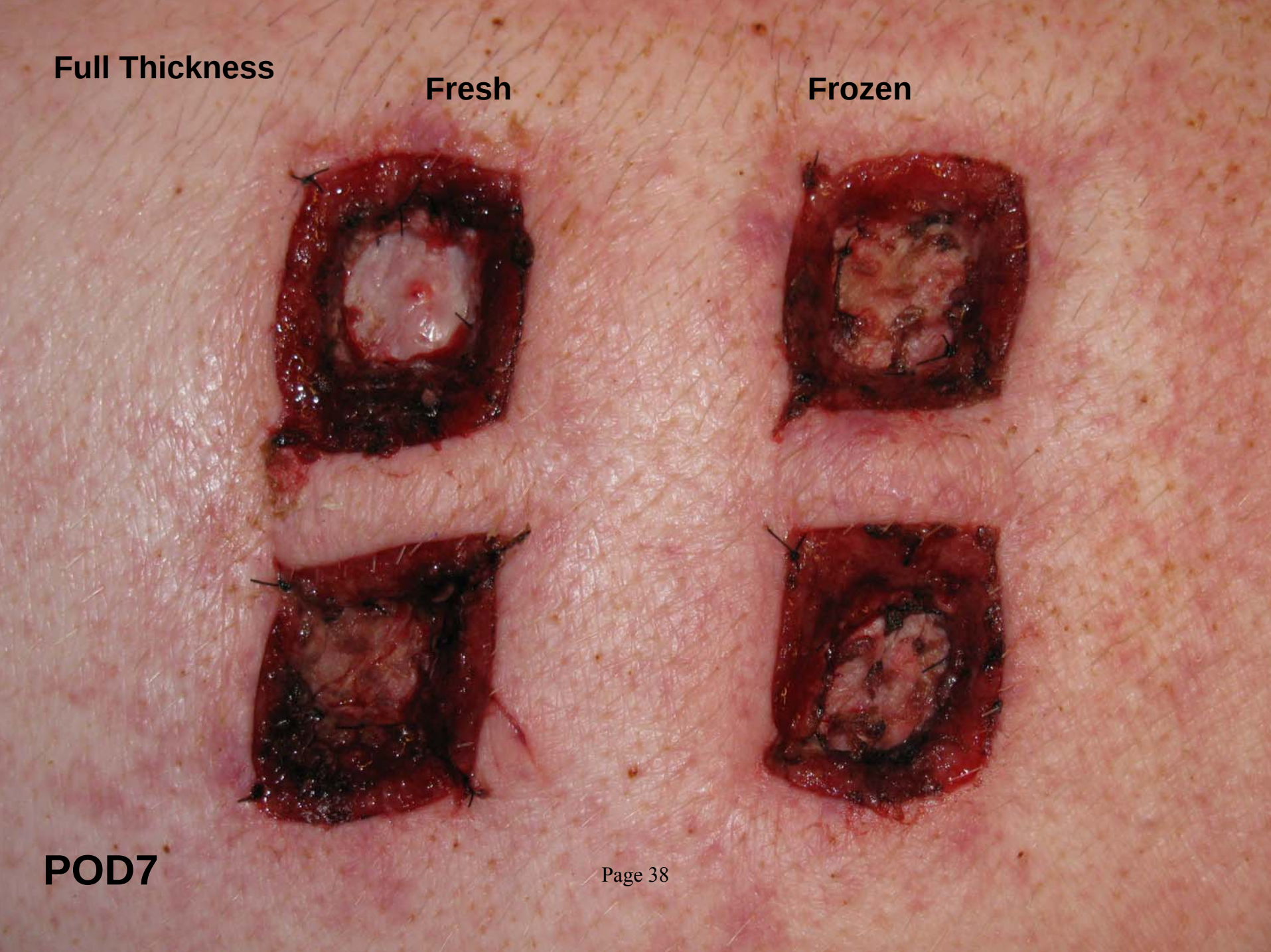
Full Thickness

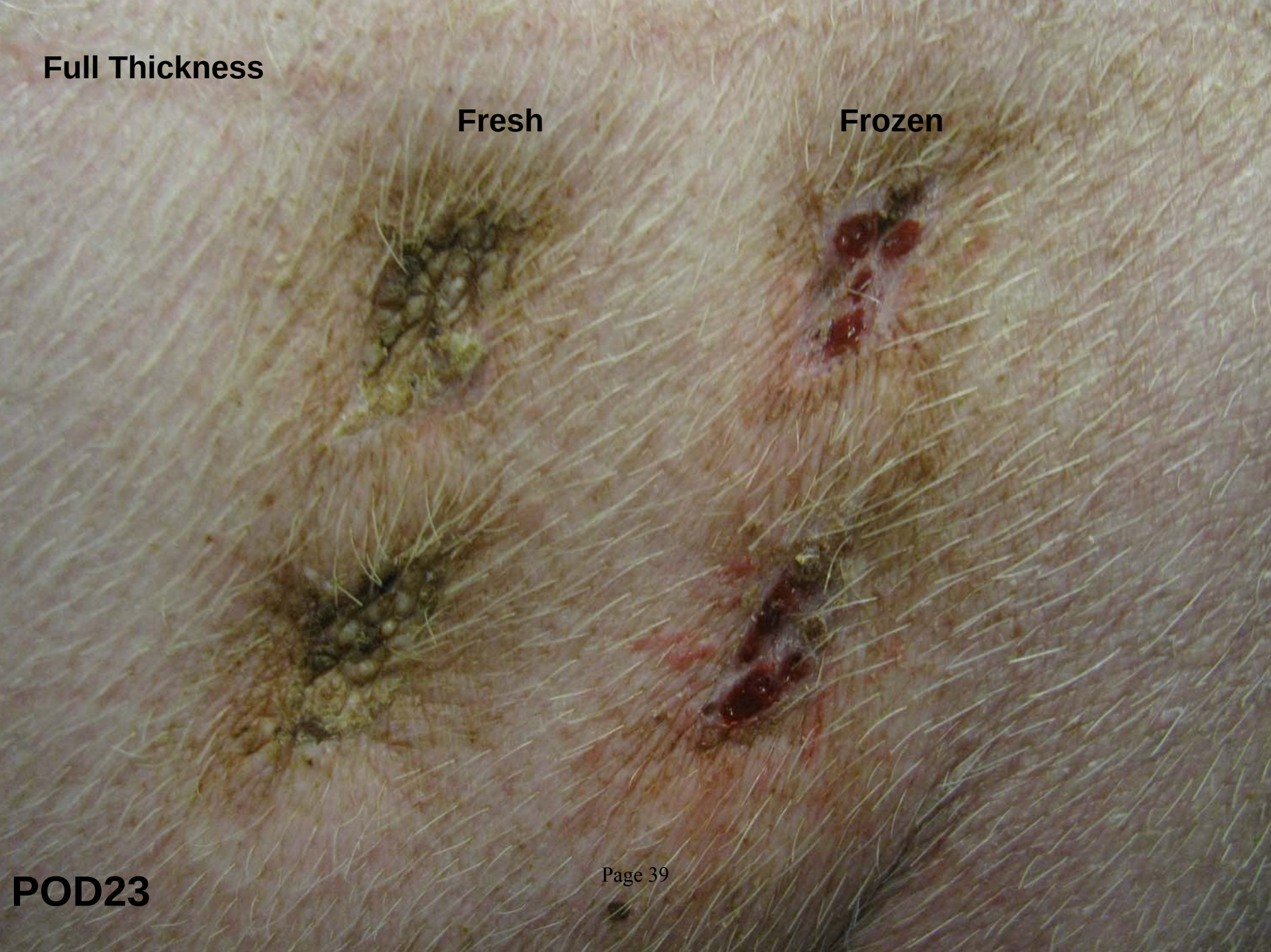


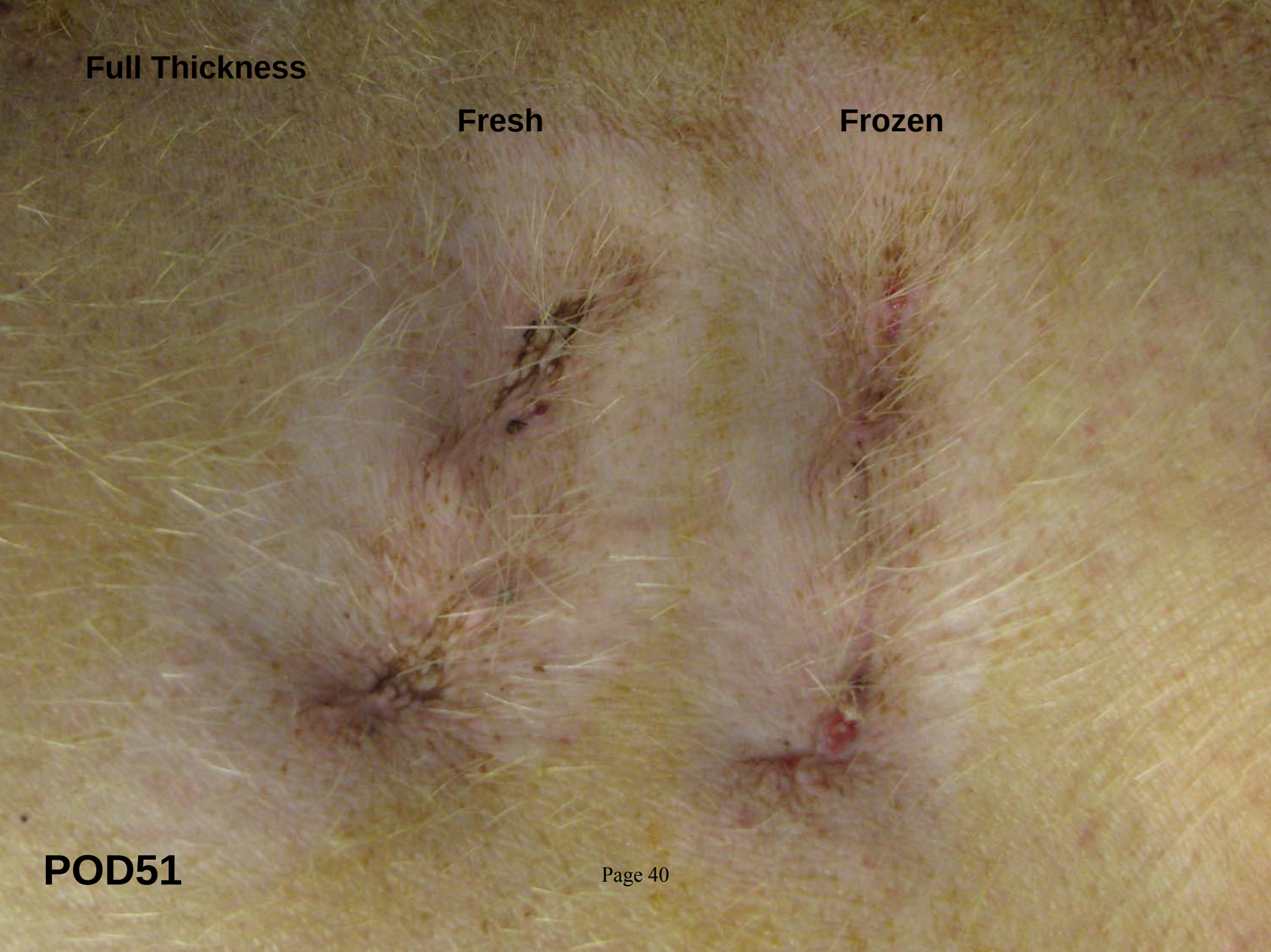
Full Thickness



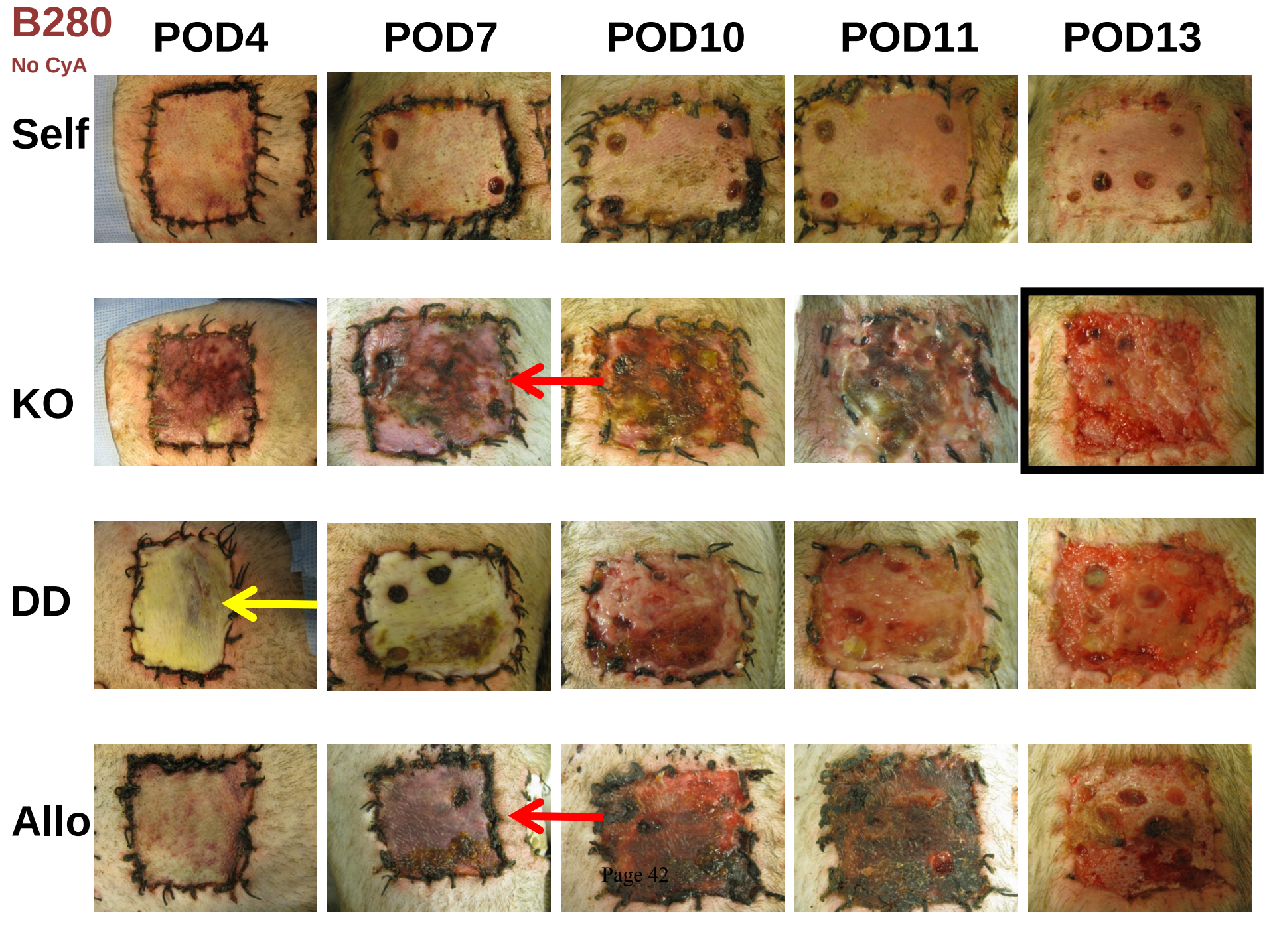

Full Thickness
Fresh
Frozen
POD3

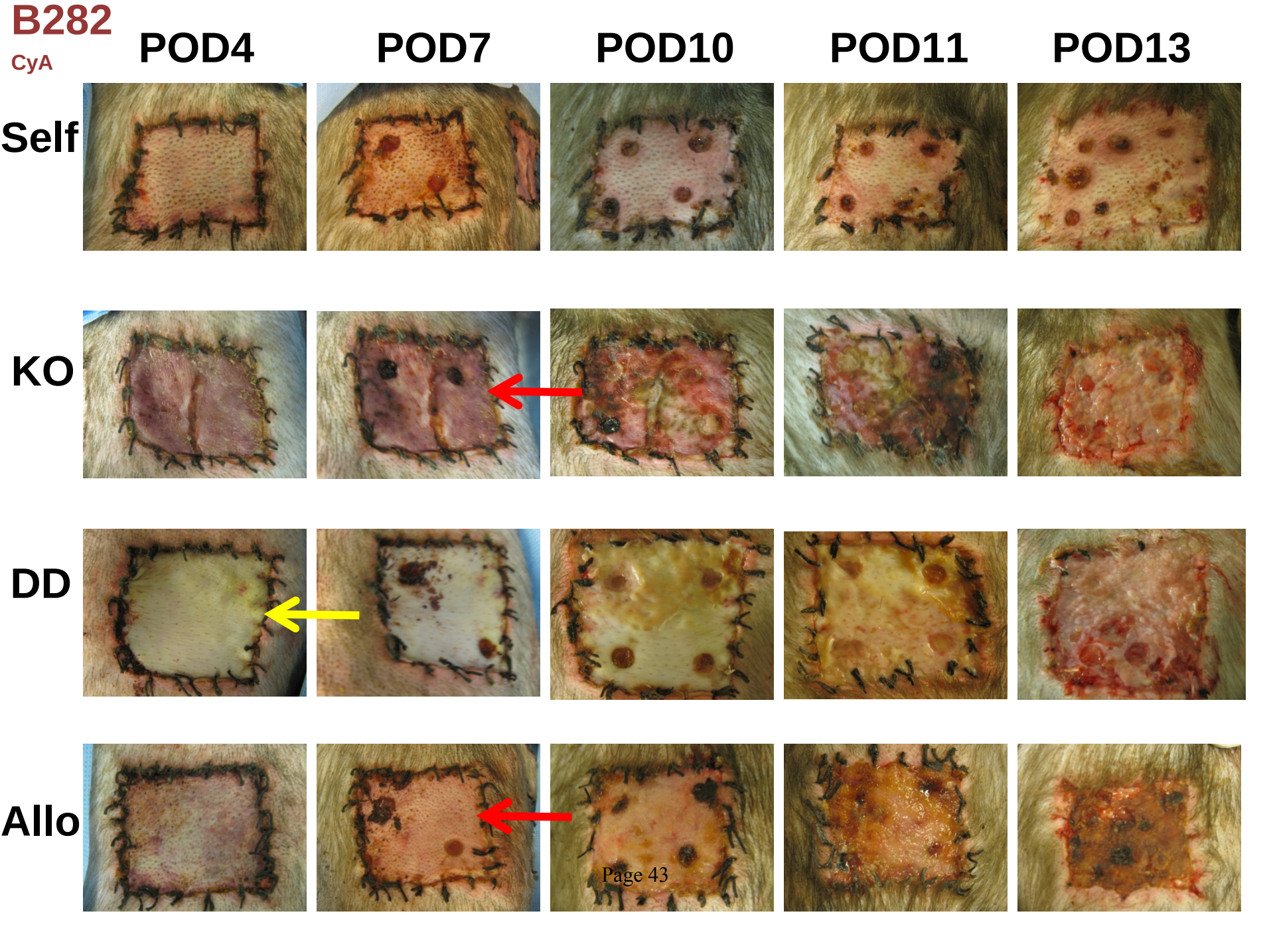

Full Thickness
Fresh
Frozen
POD7


Full Thickness
Fresh
Frozen
POD23


Full Thickness
Fresh
Frozen
POD51

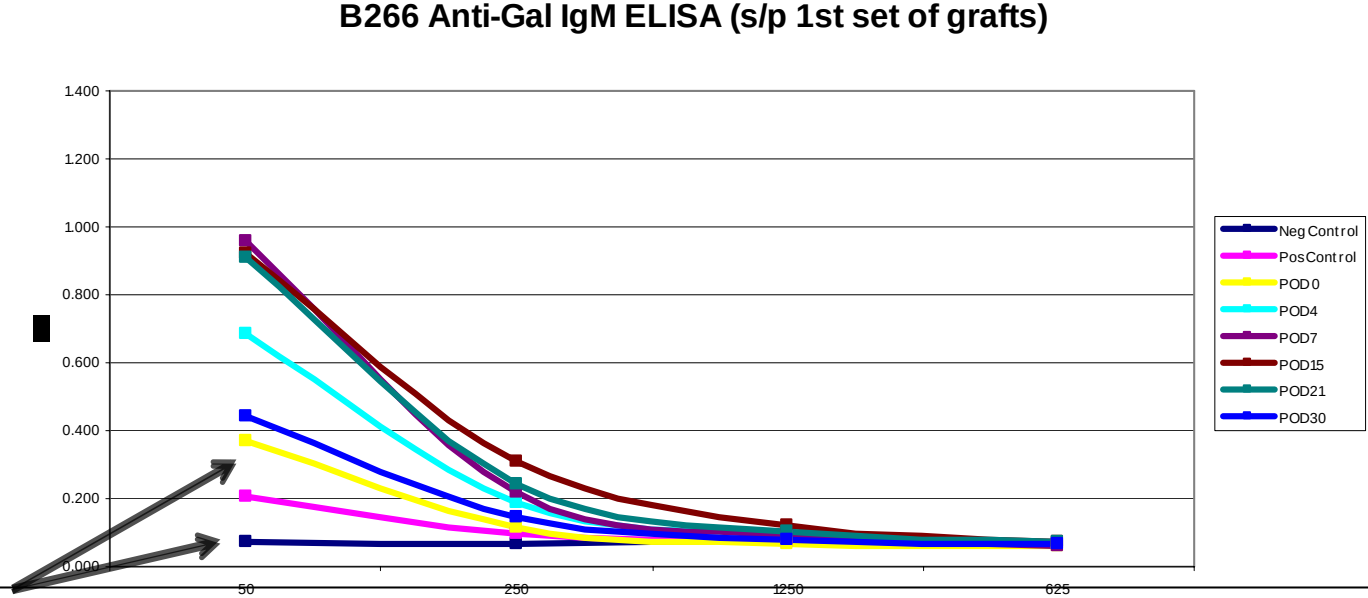
On Full-Thickness Skin Wounds


B280
No CyA
POD4
POD7
POD10
POD11
POD13
Self
KO
DD
Allo

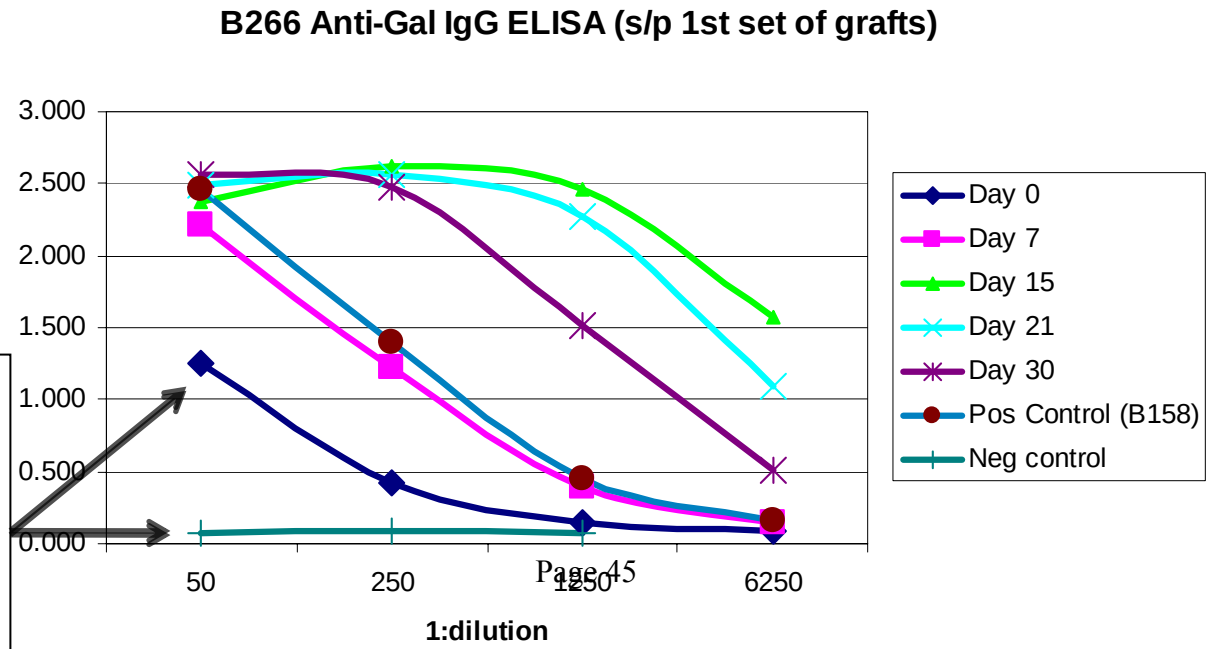

B282
CyA
POD4
POD7
POD10
POD11
POD13
Self
KO
DD
Allo


XENO SKIN GRAFTS
No CyA IM
SELF fresh
SELF frozen
B283
Allo fresh
Allo frozen
GalT- KO fresh
GalT- KO frozen
No CyA IM
SELF fresh
SELF frozen
B285
Allo fresh
Allo frozen
Gal + fresh
Gal + frozen

Figure 1



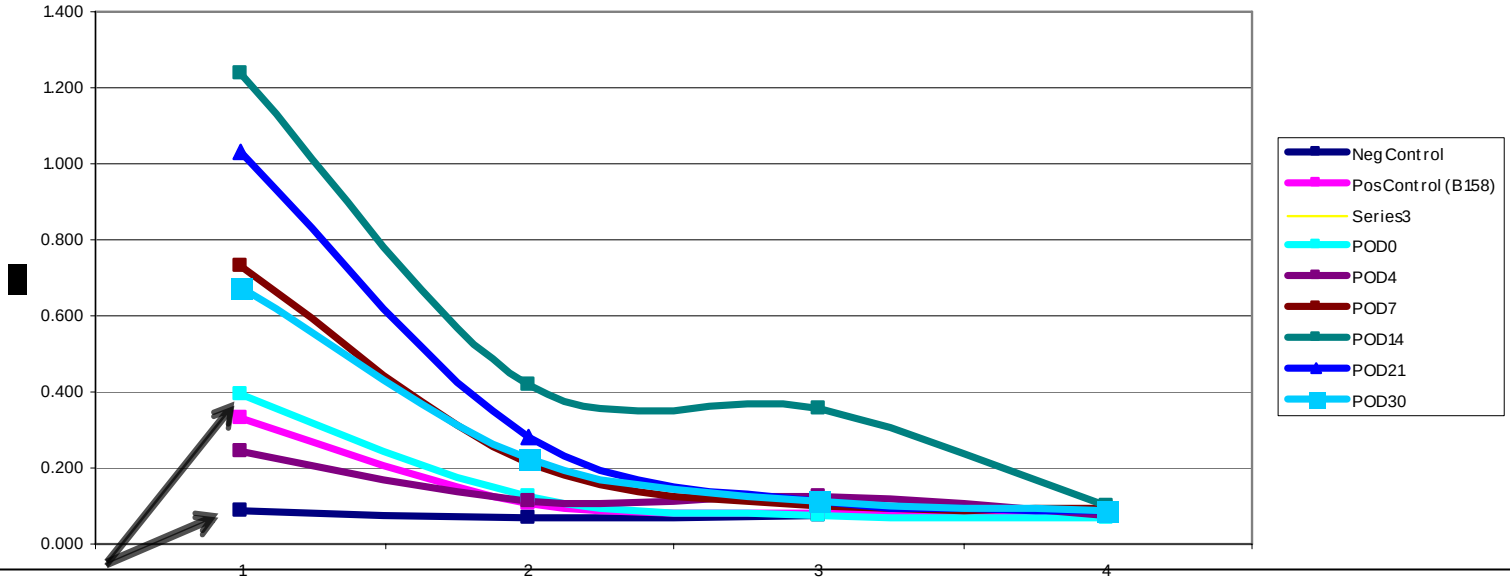
Levels of Anti-Gal IgM were present at high levels preoperatively (vs. blue bar- negative control)



Preoperative Anti-Gal IgG levels were also elevated preoperatively (vs. grey bar- negative control)

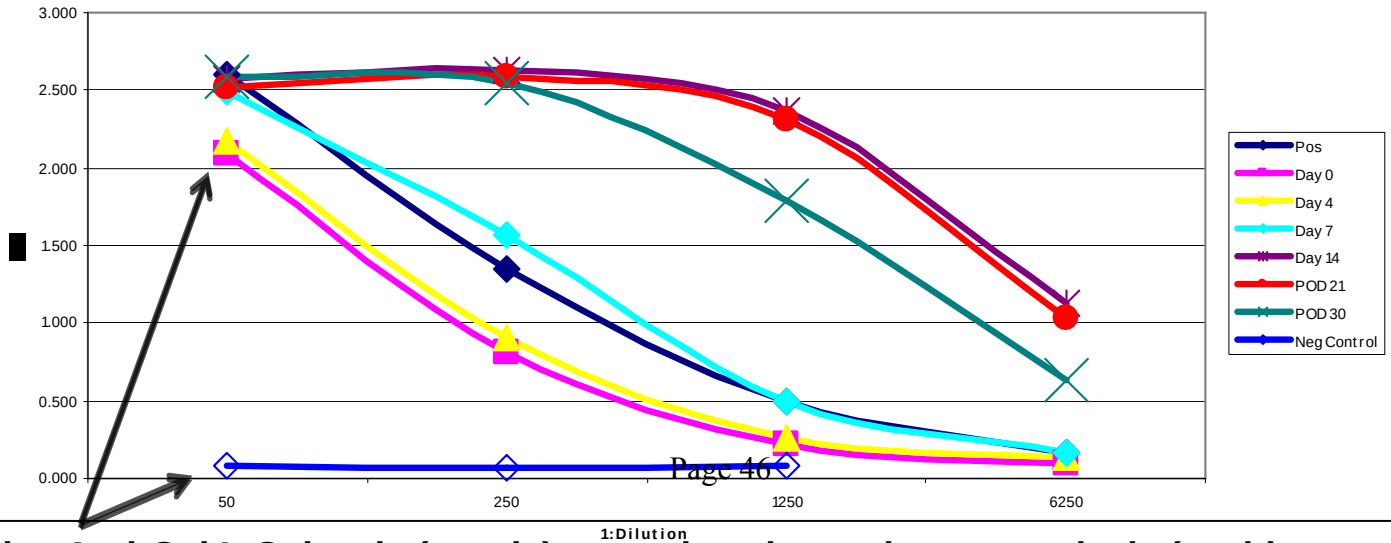
Figure 2

B267 Anti-Gal IgM ELISA (s/p 1st set of grafts)



Levels of Anti-Gal IgM (light blue) were present at high levels preoperatively (vs. blue- negative control)

B267 Anti-Gal IgG ELISA (s/p 1st set of grafts)



Preoperative Anti-Gal IgG levels (purple) were also elevated preoperatively (vs. blue-negative

Figure 3

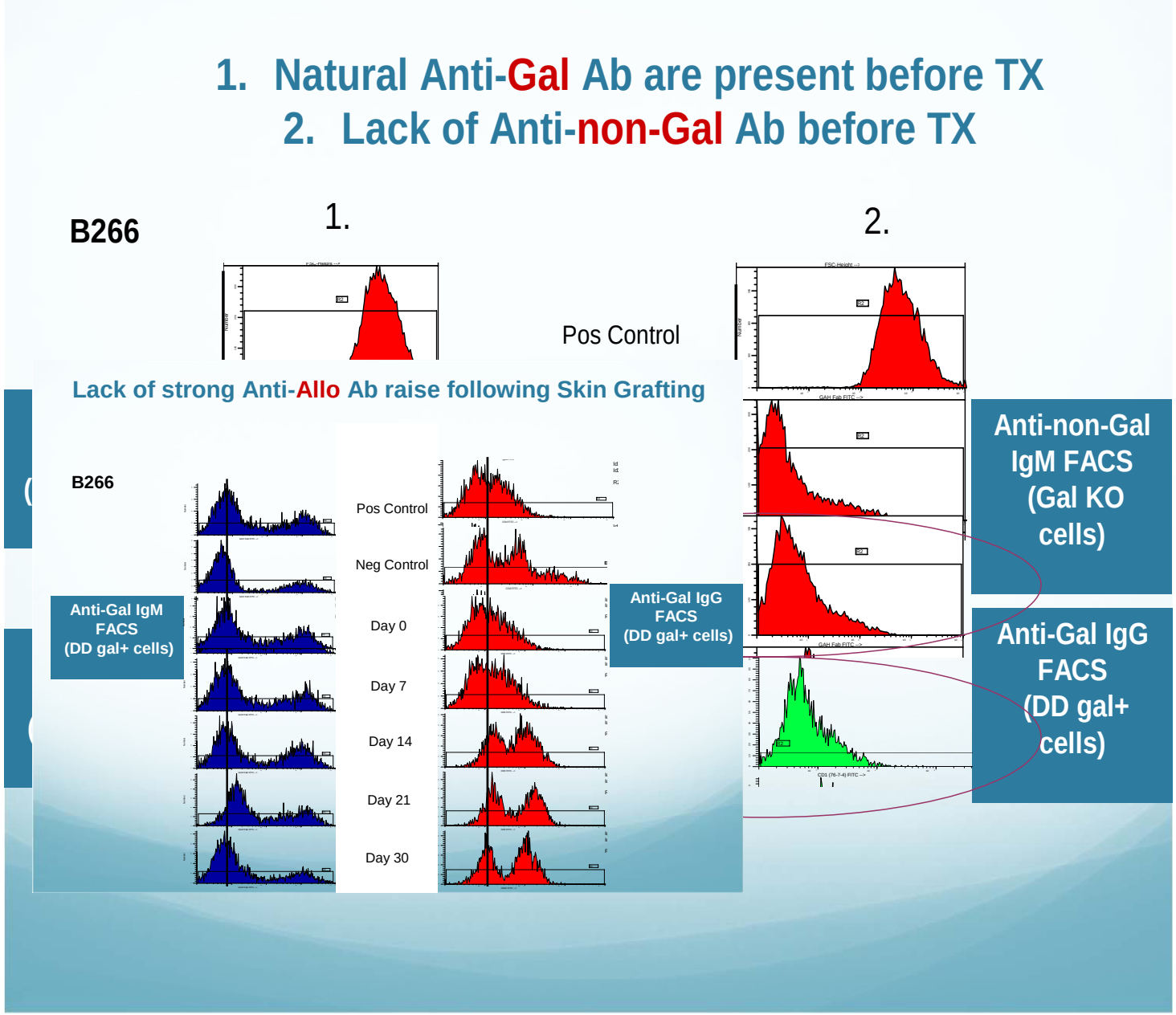
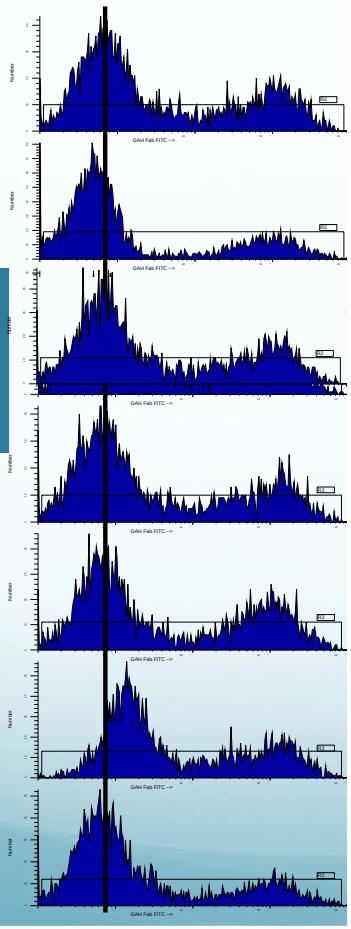

1. Natural Anti-Gal Ab are present before TX
2. Lack of Anti-non-Gal Ab before TX
B266
1.
2.
Pos Control
Lack of strong Anti-Allo Ab raise following Skin Grafting
B266
Pos Control
Neg Control
Day 0
Day 7
Day 14
Day 21
Day 30
Anti-Gal IgM FACS (DD gal+ cells)
Anti-Gal IgG FACS (DD gal+ cells)
Anti-non-Gal IgM FACS (Gal KO cells)
Anti-Gal IgG FACS (DD gal+ cells)

Figure 4

Lack of strong Anti-Allo Ab raise following Skin Grafting

B266

Anti-Gal IgM FACS (DD gal+ cells)



Pos Control

Neg Control

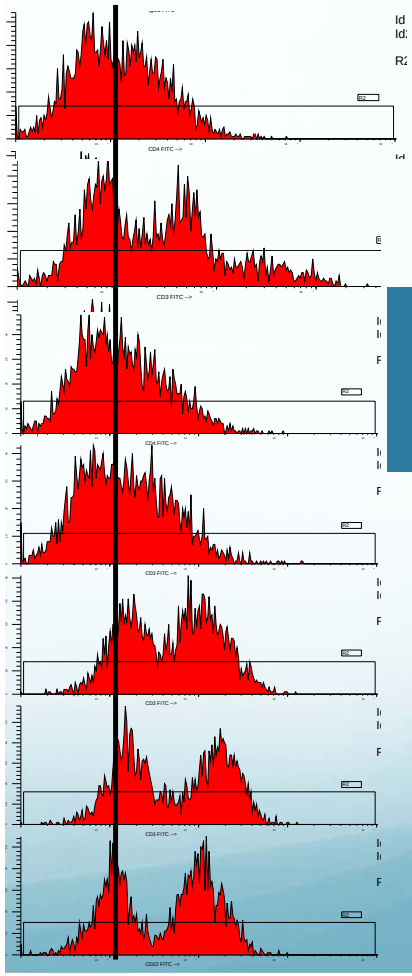
Day 0

Day 7

Day 14

Day 21

Day 30



Anti-Gal IgG FACS (DD gal+ cells)

Figure 5

Allo reactivity is greater **pre** skin transplant
Xeno reactivity is greater **post** skin transplant

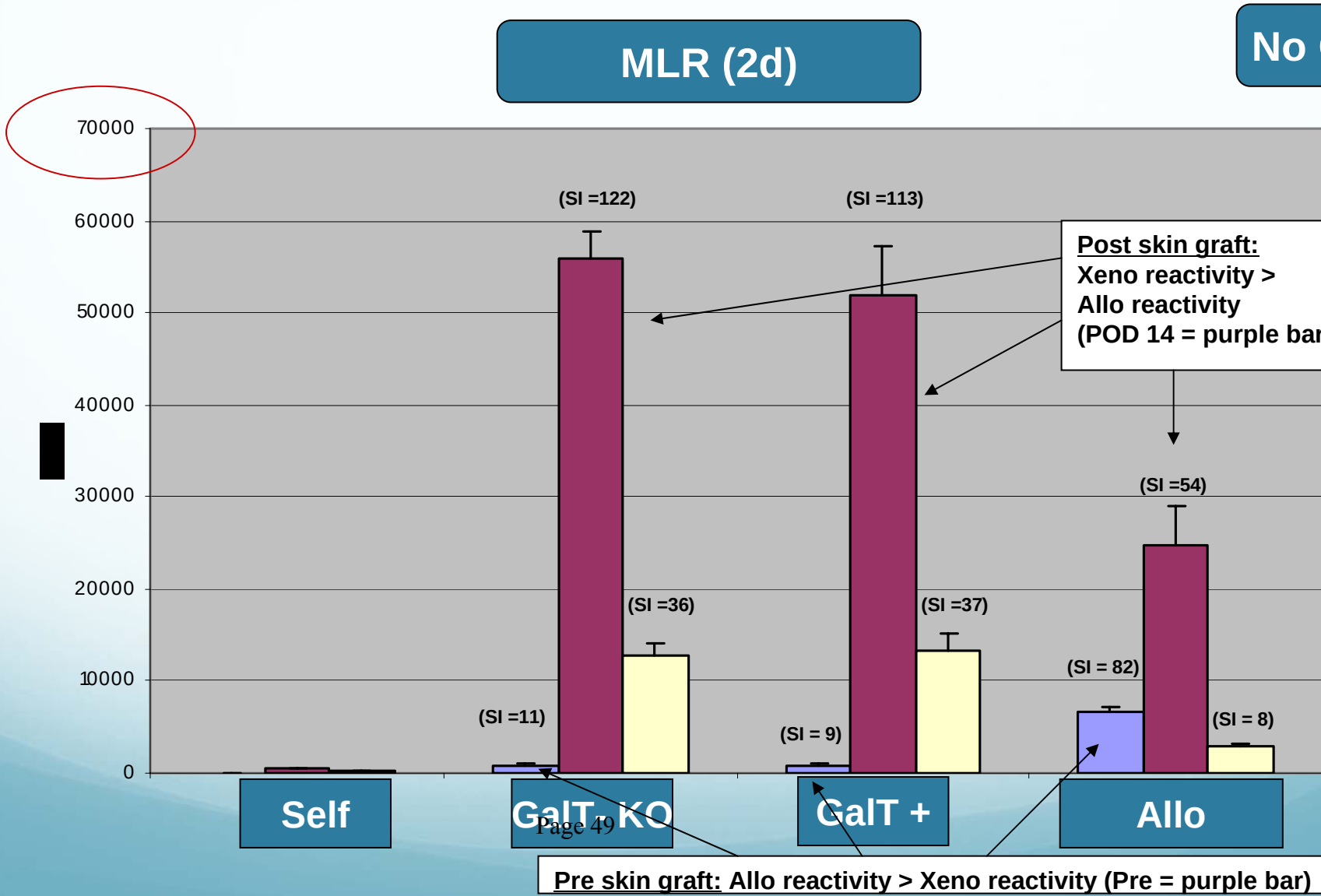
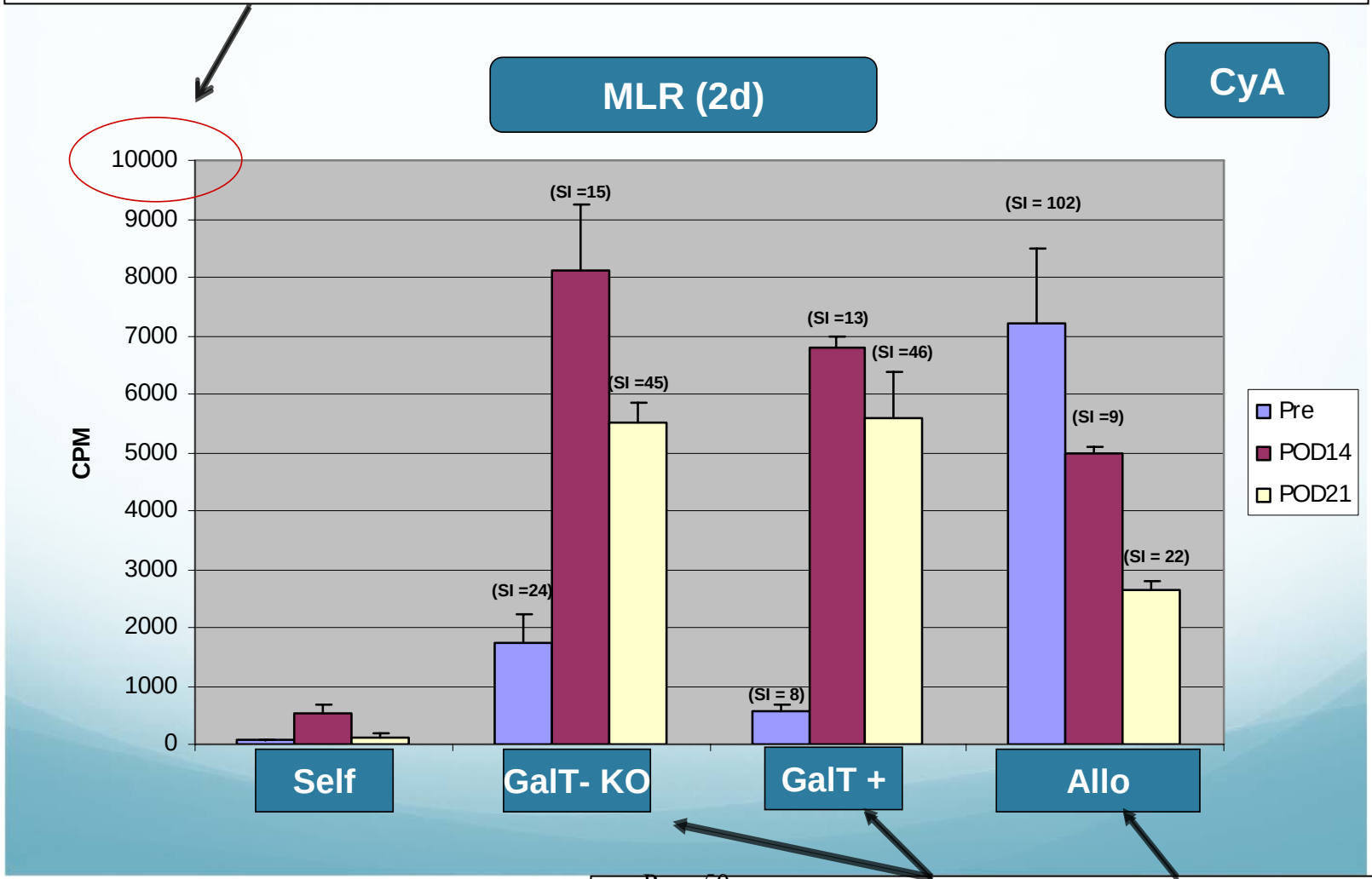


Figure 6

Cyclosporine A suppressed both Xeno and Allo T cell responses



CyA suppressed both Xeno and Allo responses (as shown by lower cpm (counts per minutes) (vs. Figure 5)

Figure 7

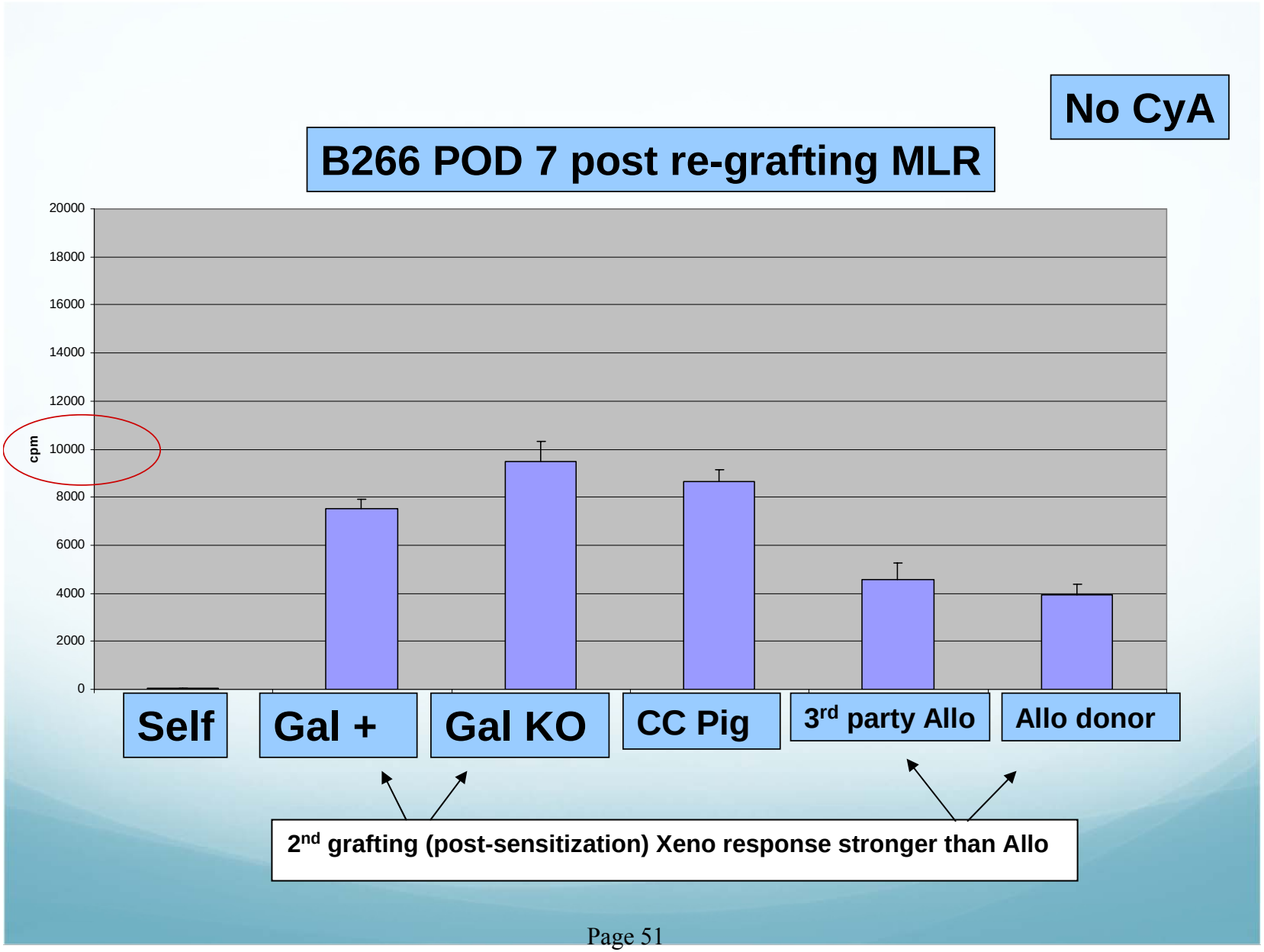

No CyA
B266 POD 7 post re-grafting MLR
cpm
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Self
Gal +
Gal KO
CC Pig
3rd party Allo
Allo donor
2nd grafting (post-sensitization) Xeno response stronger than Allo

Figure 8

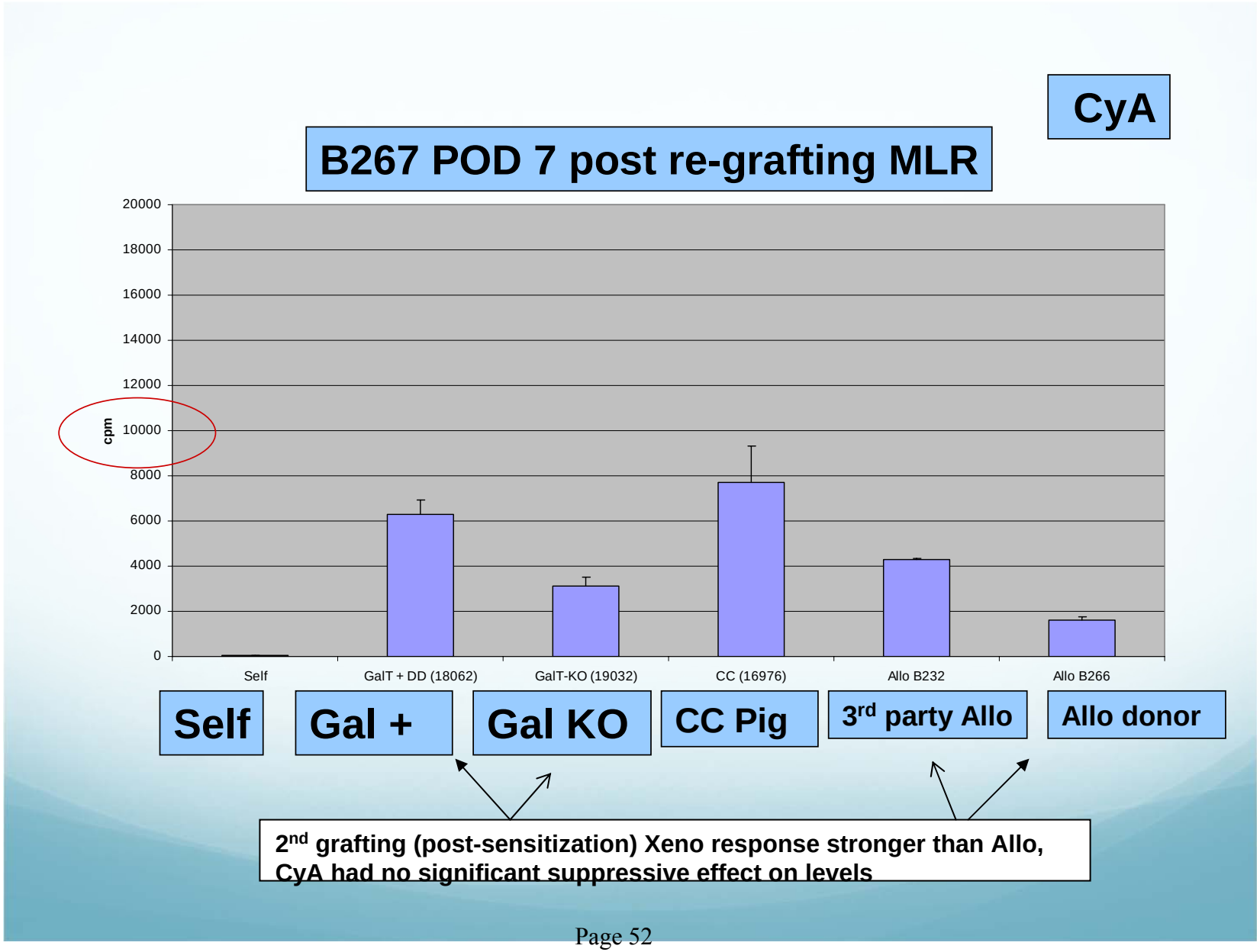

CyA
B267 POD 7 post re-grafting MLR
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
cpm
Self
GalT + DD (18062)
GalT-KO (19032)
CC (16976)
Allo B232
Allo B266
Self
Gal +
Gal KO
CC Pig
3rd party Allo
Allo donor
2nd grafting (post-sensitization) Xeno response stronger than Allo, CyA had no significant suppressive effect on levels

Figure 1

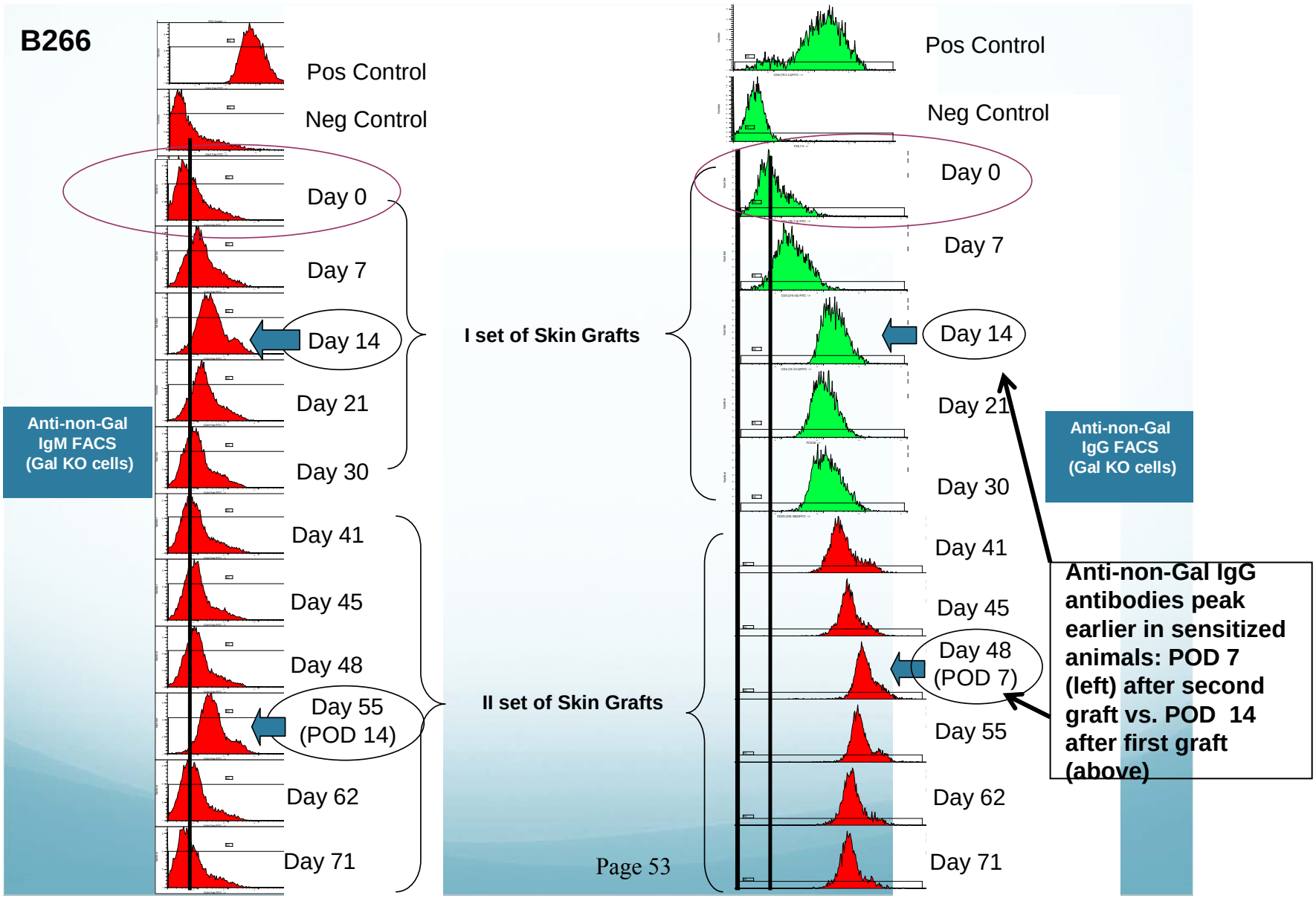

B266
Pos Control
Neg Control
Day 0
Day 7
Day 14
Day 21
Day 30
Day 41
Day 45
Day 48
Day 55
(POD 14)
Day 62
Day 71
Anti-non-Gal
IgM FACS
(Gal KO cells)
I set of Skin Grafts
II set of Skin Grafts
Pos Control
Neg Control
Day 0
Day 7
Day 14
Day 21
Day 30
Day 41
Day 45
Day 48
(POD 7)
Day 55
Day 62
Day 71
Anti-non-Gal
IgG FACS
(Gal KO cells)
Anti-non-Gal IgG antibodies peak earlier in sensitized animals: POD 7 (left) after second graft vs. POD 14 after first graft (above)

Figure 1

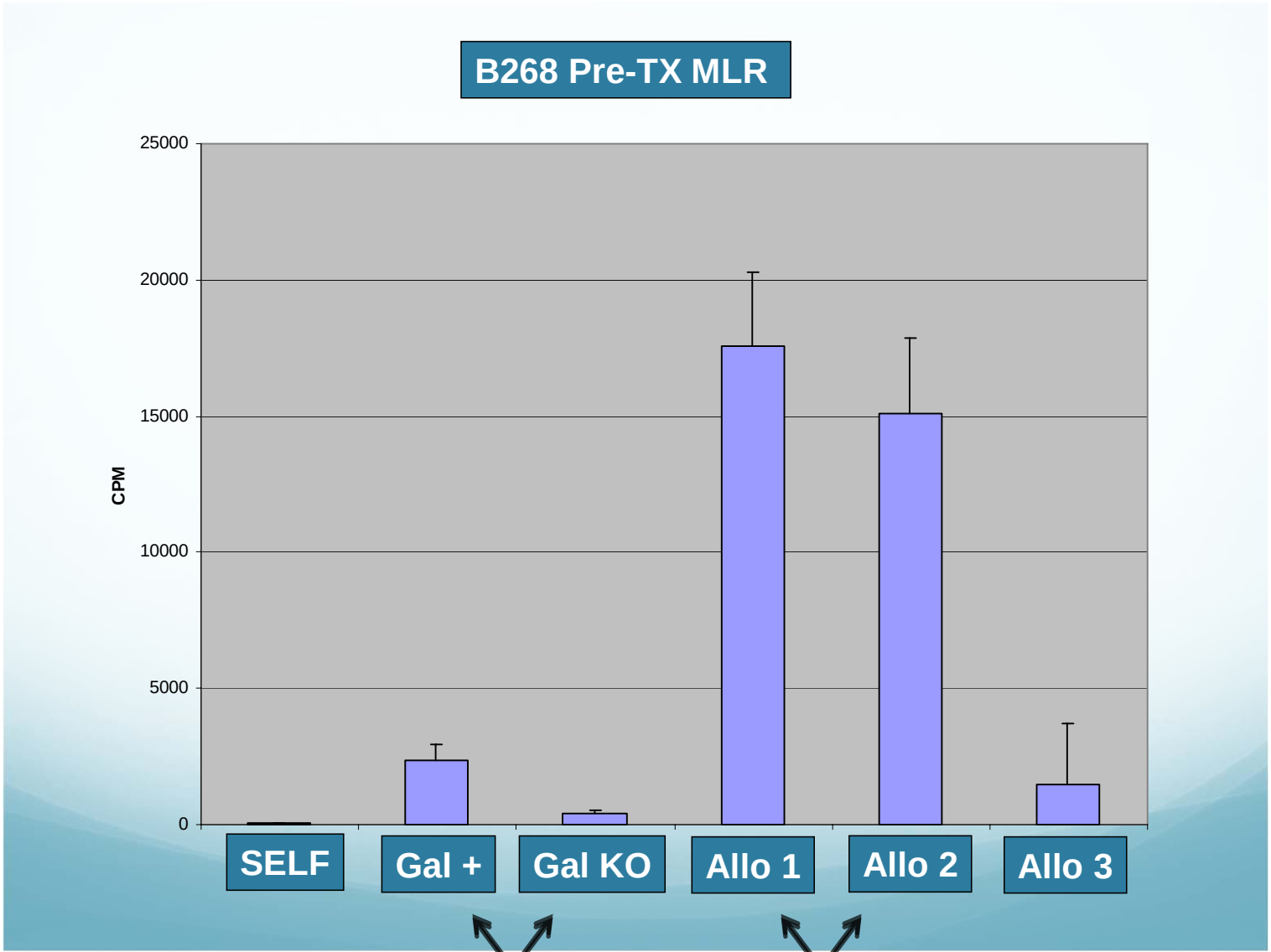
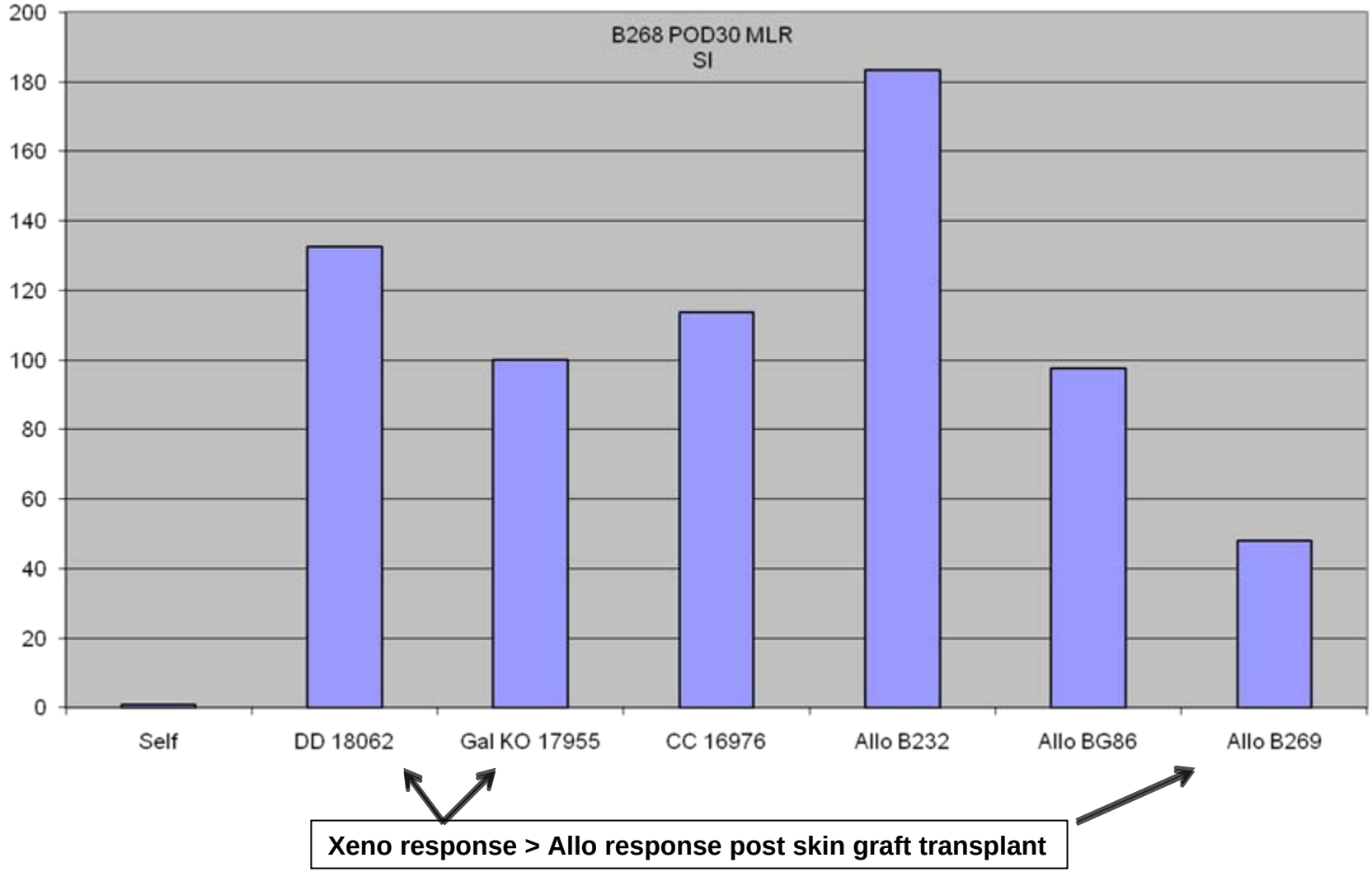
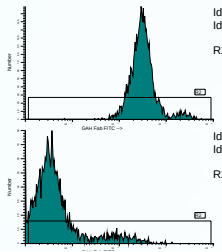

B268 Pre-TX MLR
25000
20000
15000
10000
5000
0
CPM
SELF
Gal +
Gal KO
Allo 1
Allo 2
Allo 3
Allo response > Xeno (Gal+, GalT-KO) response pre skin graft transplant

Figure 2


B268 POD30 MLR
SI
200
180
160
140
120
100
80
60
40
20
0
Self
DD 18062
Gal KO 17955
CC 16976
Allo B232
Allo BG86
Allo B269
Xeno response > Allo response post skin graft transplant

B268 Anti-nonGal IgM and IgG FACS



Id1: B268B269.015
Id2: 9Dec2009

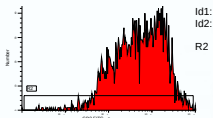
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	411.05	283.89	305.07

Pos Control

Id1: B268B269.016
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	91.21	18.85	3.65	3.79

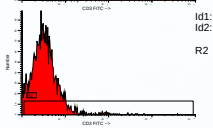
Neg Control (FPS)



Id1: 015.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.91	957.22	1715.55	562.34

Pos Control

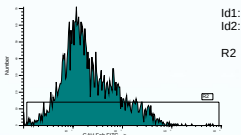


Id1: 016.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	93.26	7.68	2.74	3.28

Neg Control (FPS)

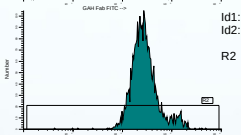
IgM



Id1: B268B269.019
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.76	60.60	11.55	17.16

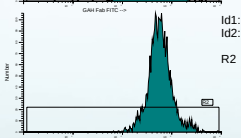
Day 0



Id1: B268B269.020
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	355.23	264.18	264.18

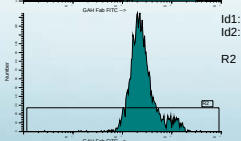
Day 7



Id1: B268B269.021
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	670.53	453.19	562.34

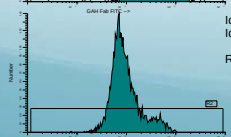
Day 14



Id1: B268B269.022
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	318.04	198.11	237.15

Day 21

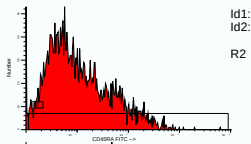


Id1: B268B269.023
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	127.31	69.79	83.54

Day 30

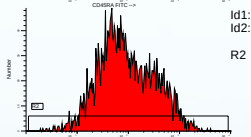
IgG



Id1: 019.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	97.43	35.44	5.62	8.66

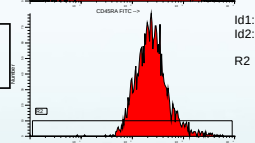
Day 0



Id1: 020.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	198.89	46.98	80.59

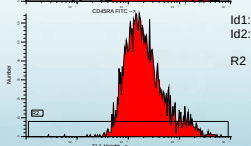
Day 7



Id1: 021.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.97	323.84	228.76	237.15

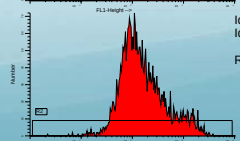
Day 14



Id1: 022.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	336.66	138.24	184.35

Day 21



Id1: 023.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	286.37	107.46	133.36

Day 30

Anti-nonGal Abs peak at POD 14

B268 Anti-Allo IgM and IgG FACS

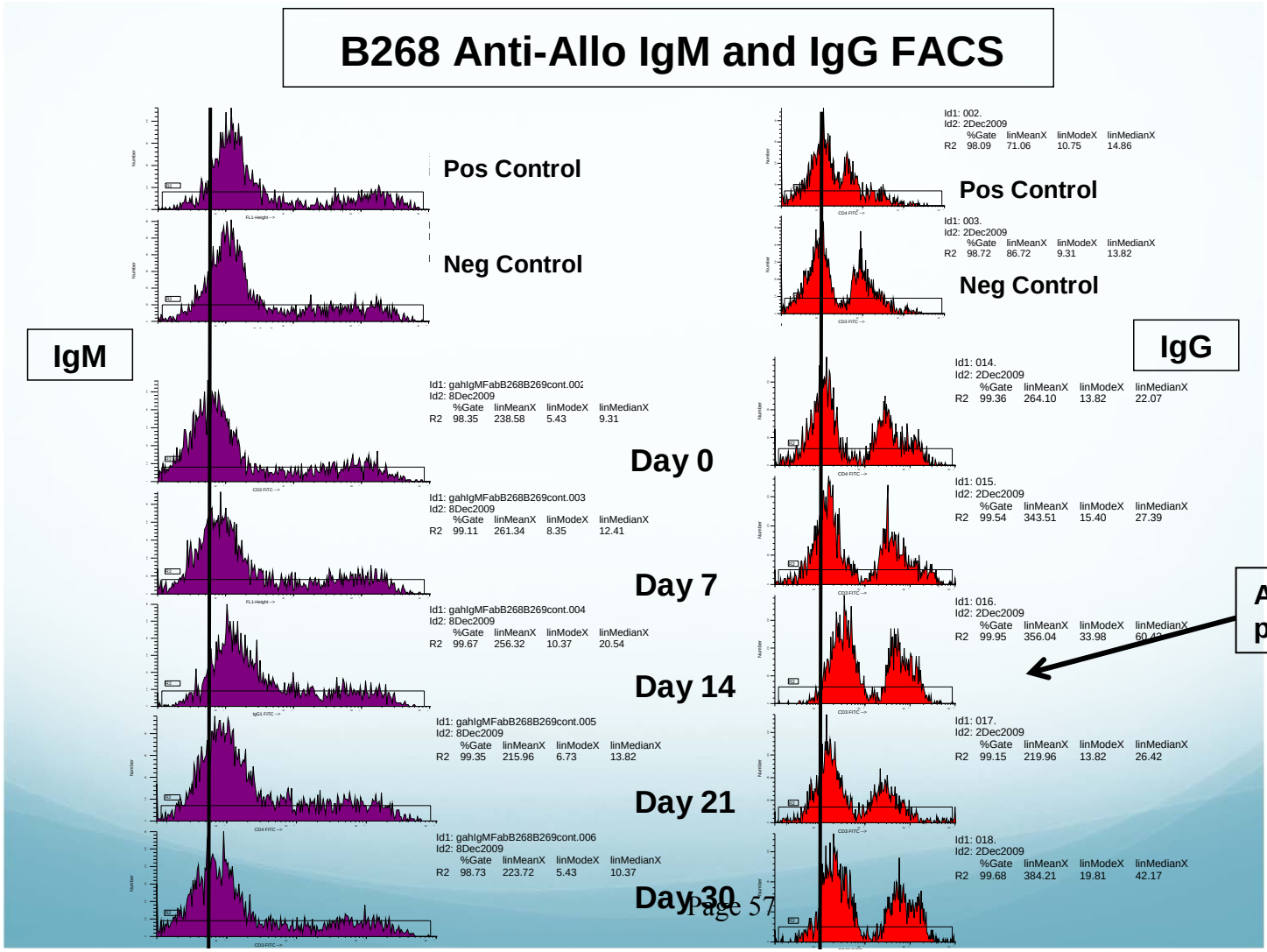


Figure 1

B269 Anti-nonGal IgM and IgG FACS

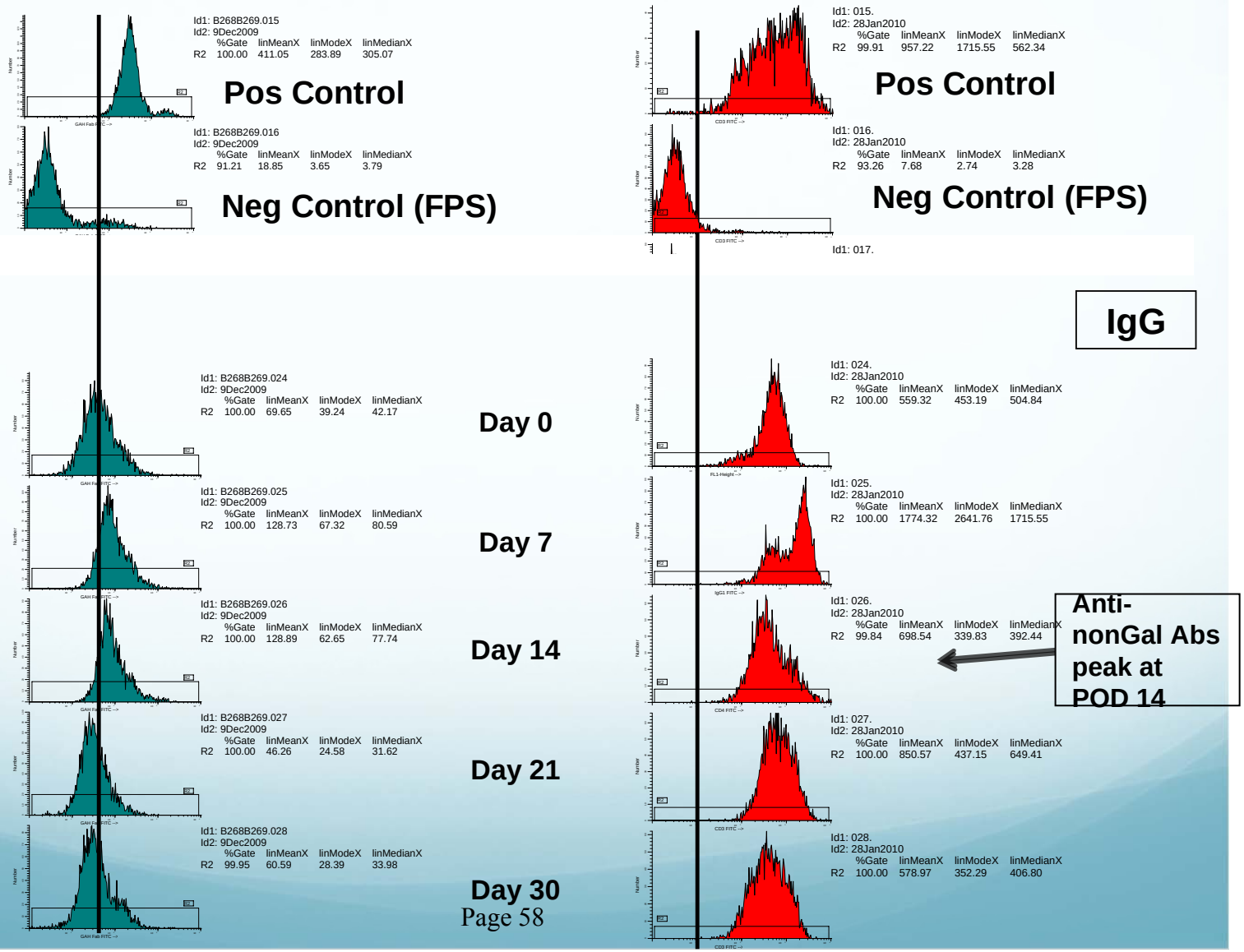


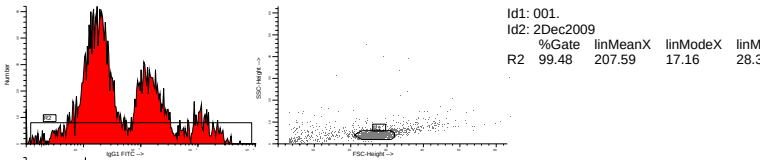
Figure 2

Anti-Allo IgG Fab Facs

12/01/2009

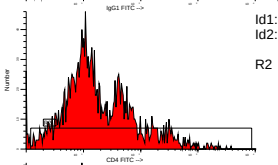
Opt log 36668

B268+B158+II



Id1: 001.
Id2: 2Dec2009

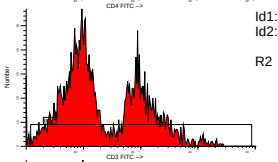
	%Gate	linMeanX	linModeX	linM
R2	99.48	207.59	17.16	28.3



Id1: 002.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.09	71.06	10.75	14.86

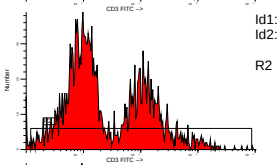
B269+158+II



Id1: 003.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.72	86.72	9.31	13.82

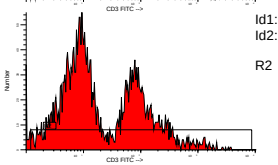
B268+FPS+II



Id1: 004.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.92	106.78	9.65	15.40

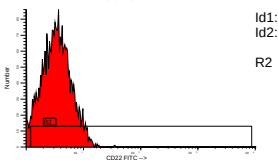
B269+FPS+II



Id1: 005.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.29	87.55	9.31	13.34

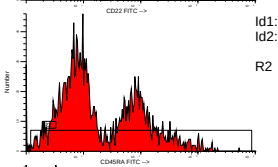
B268+II



Id1: 006.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	92.85	4.10	3.65	3.52

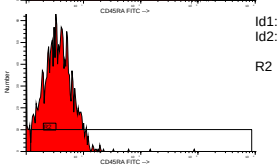
B268 cells alone



Id1: 007.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.50	81.53	9.65	11.97

B269+II

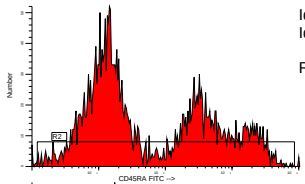


Id1: 008.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.01	4.78	3.28	3.65

B269 cells alone

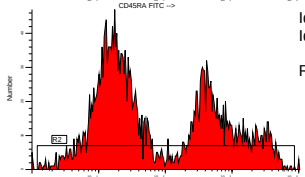
B269



Id1: 009.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.58	398.71	14.33	25.48

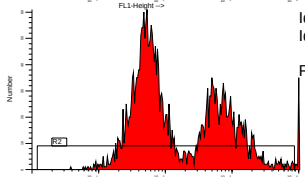
Day 0



Id1: 010.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.64	483.68	17.16	36.52

Day 7

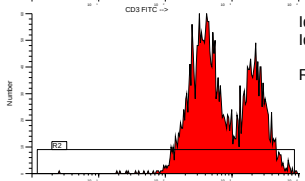


Id1: 011.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.69	460.27	52.33	86.60

Day 14

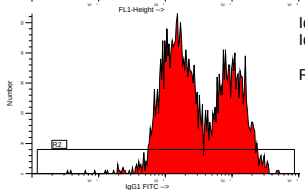
Anti-Allo Abs peak at POD 14



Id1: 012.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	1171.73	316.23	582.98

Day 21



Id1: 013.
Id2: 2Dec2009

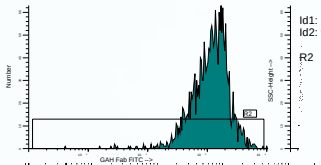
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	562.13	148.56	264.18

Day 30

Figure 3

B269 Anti-Gal IgM and IgG FACS

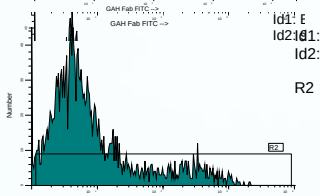
IgM



Id1: B268B269.001
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.95	1371.58	1654.86	1241.01

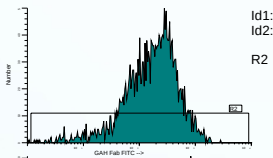
Pos Control



Id1: B268B269.002
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	95.49	59.28	4.22	5.83

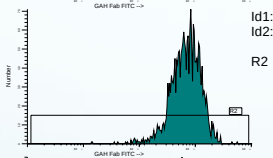
Neg Control (FPS)



Id1: B268B269.010
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.88	254.59	273.86	191.10

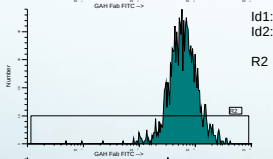
Day 0



Id1: B268B269.011
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	785.85	805.89	723.41

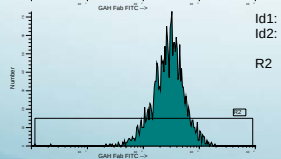
Day 7



Id1: B268B269.012
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	705.33	562.34	604.31

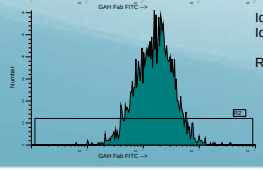
Day 14



Id1: B268B269.013
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	333.99	316.23	294.28

Day 21

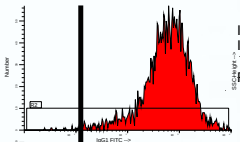


Id1: B268B269.014
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	209.66	154.00	165.49

Day 30

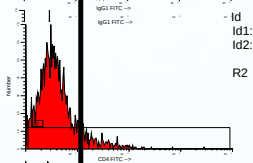
IgG



Id1: 001.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.97	855.94	604.31	626.47

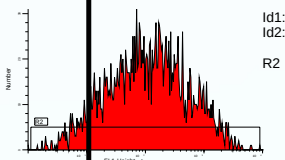
Pos Control



Id1: 002.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	93.57	8.70	3.05	3.65

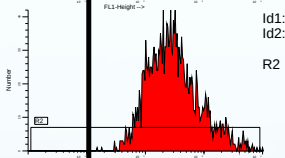
Neg Control (FPS)



Id1: 010.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.92	308.30	69.79	111.40

Day 0

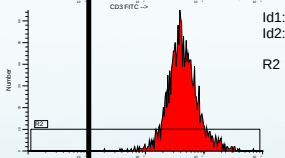


Id1: 011.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.91	531.65	198.11	273.86

Day 7

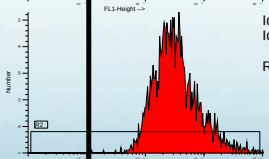
Anti-Gal Abs peak at POD 7



Id1: 012.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	526.15	378.57	421.72

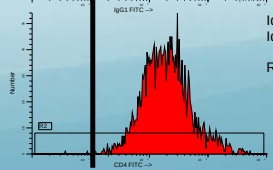
Day 14



Id1: 013.
Id2: 28Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.92	522.87	365.19	305.07

Day 21



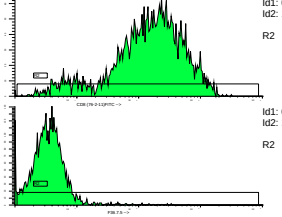
Id1: 014.
Id2: 28Jan2010

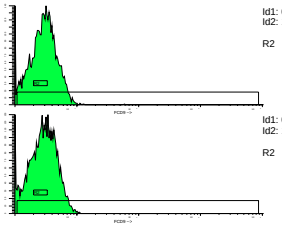
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	318.45	294.28	191.10

Day 30

Supplementary
FACS and MLR Data

Does Re-grafting Accelerate Humoral Response?



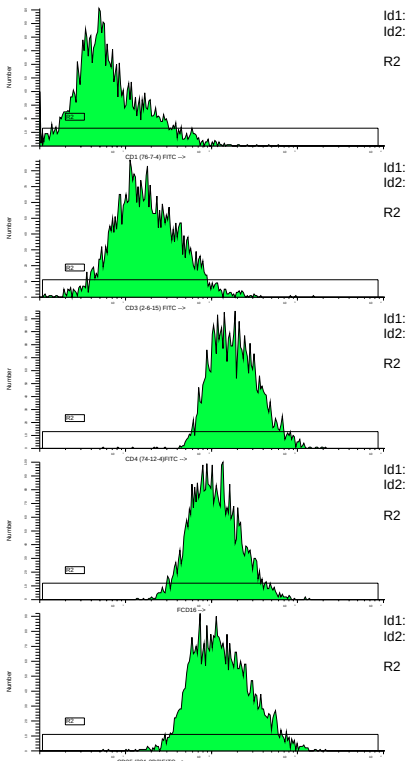


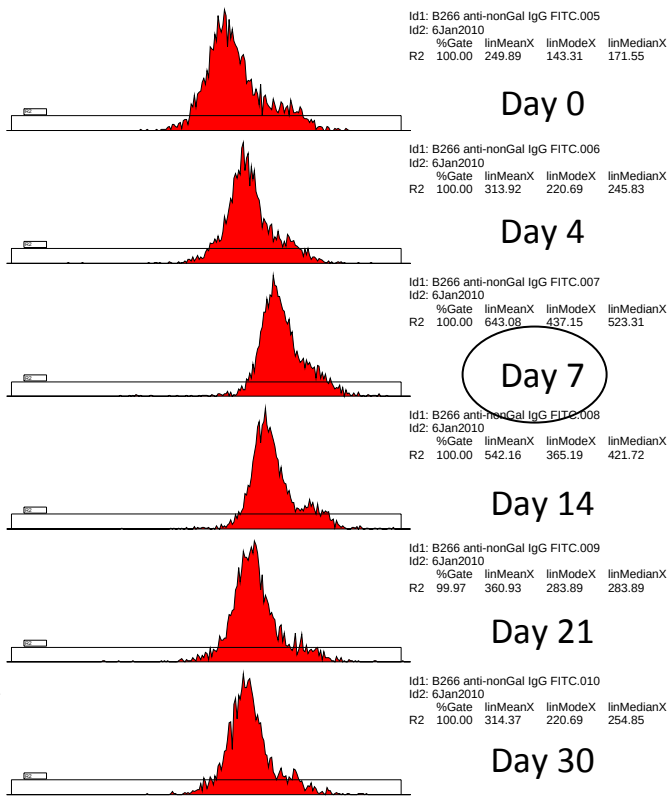
Anti-nonGal IgG FACS

I set of Skin Grafts

II set of Skin Grafts

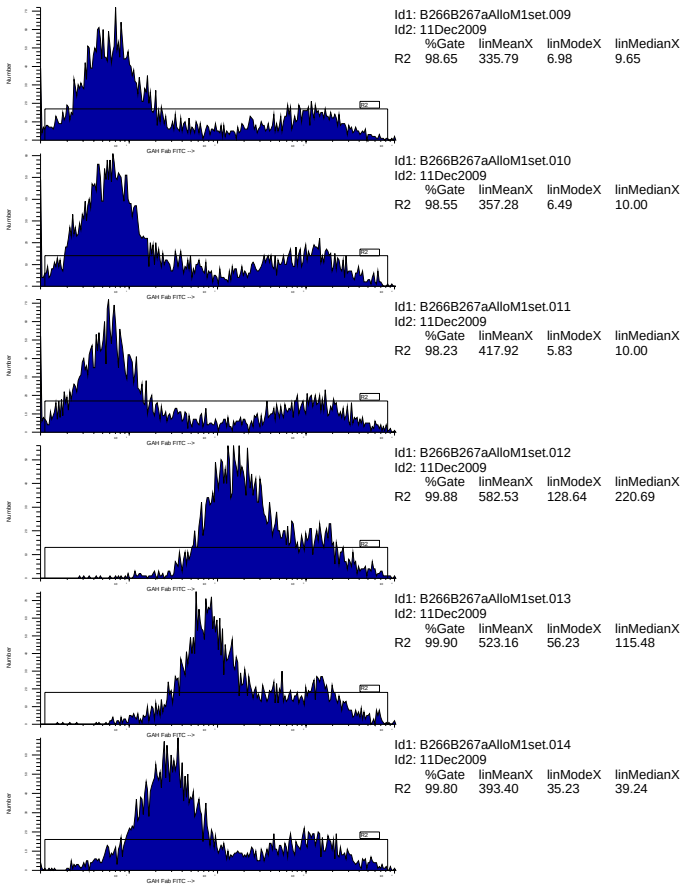
B266





B267 Anti-Allo IgM Facs

I set of skin grafts



Day 0

Day 4

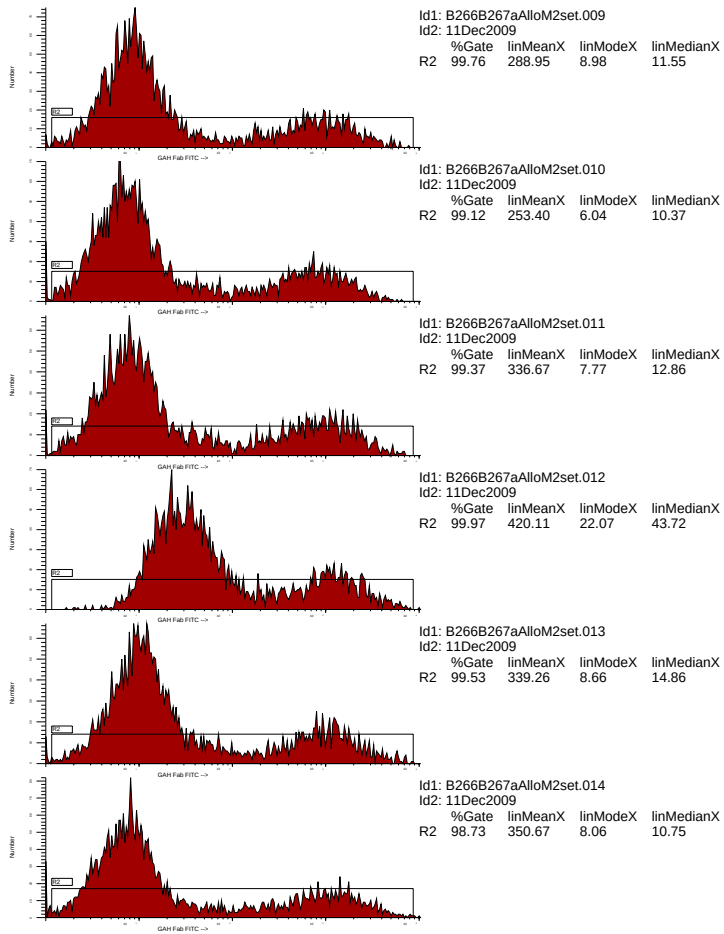
Day 7

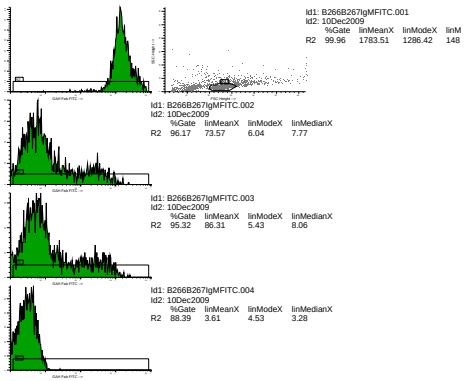
Day 14

Day 21

Day 30

II set of skin grafts



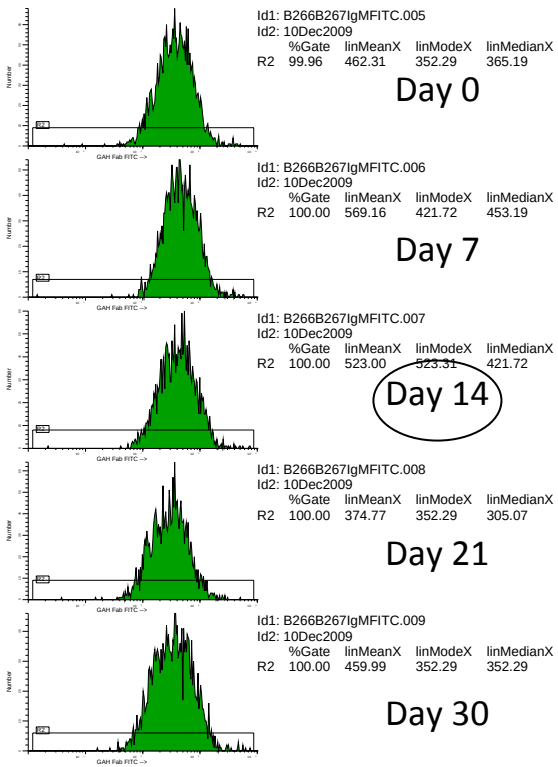


Does re-grafting accelerate humoral response?

Anti-Gal IgM FACS

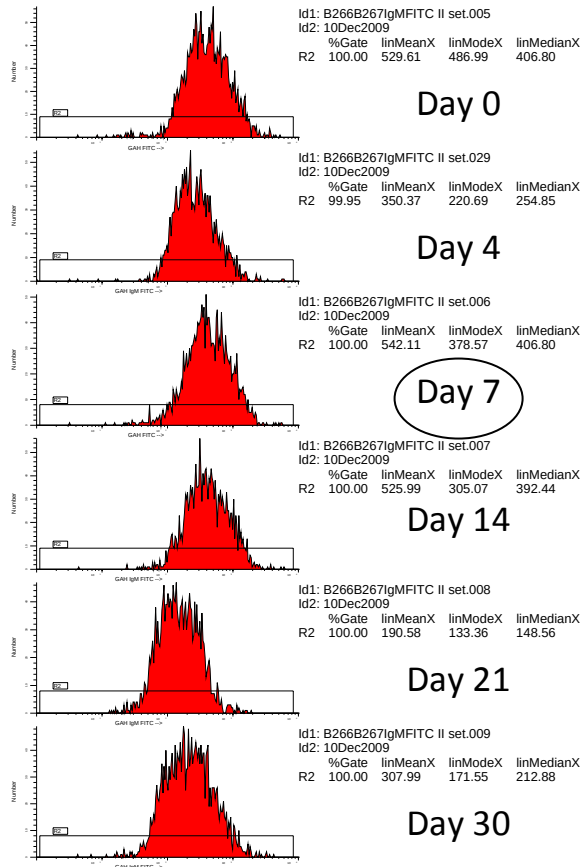
I set of Skin Grafts

B266

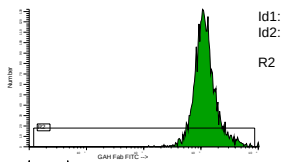


II set of Skin Grafts

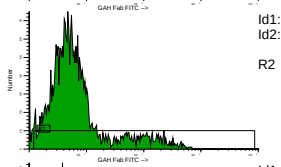
B266



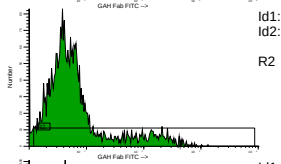
Anti-nonGal IgM FACS



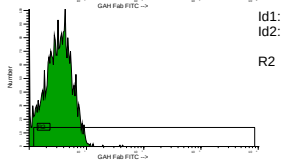
B268 POD14 (Pos Contr)



Neg Control

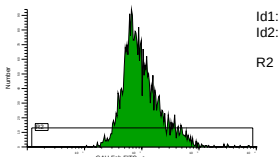


Reg DD cells + II Ab

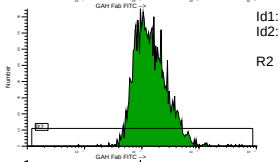


Cells alone

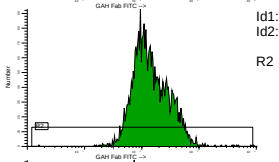
B266



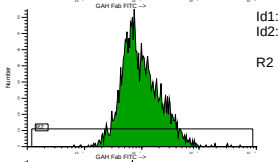
Day 0



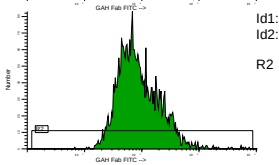
Day 7



Day 14

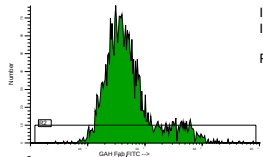


Day 21

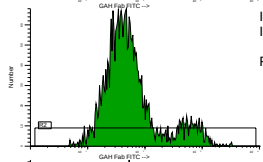


Day 30

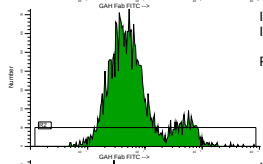
B267



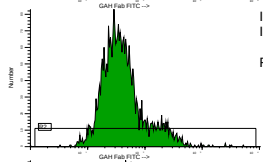
Day 0



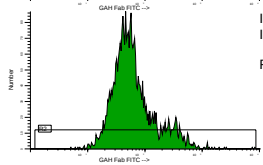
Day 7



Day 14



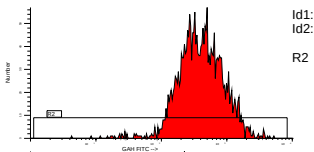
Day 21



Day 30

Anti-Gal IgM FACS Following Regrafting

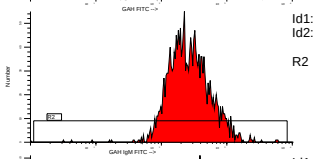
B266



Id1: B266B267IgMFITC II set.005
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	529.61	486.99	406.80

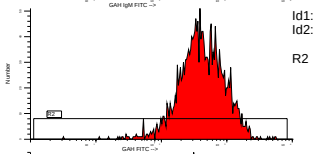
Day 0



Id1: B266B267IgMFITC II set.029
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.95	350.37	220.69	254.85

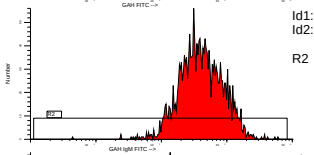
Day 4



Id1: B266B267IgMFITC II set.006
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	542.11	378.57	406.80

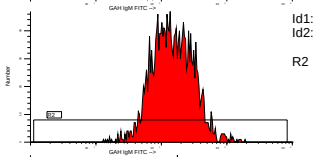
Day 7



Id1: B266B267IgMFITC II set.007
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	525.99	305.07	392.44

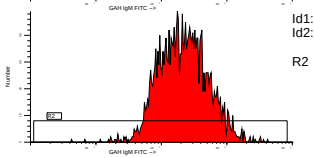
Day 14



Id1: B266B267IgMFITC II set.008
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	190.58	133.36	148.56

Day 21

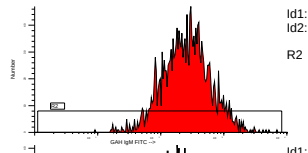


Id1: B266B267IgMFITC II set.009
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	307.99	171.55	212.88

Day 30

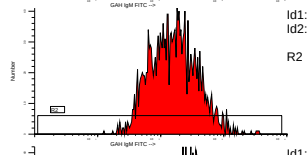
B267



Id1: B266B267IgMFITC II set.010
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	370.83	294.28	264.18

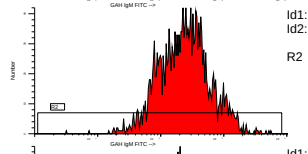
Day 0



Id1: B266B267IgMFITC II set.030
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	230.11	177.83	159.64

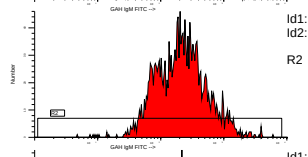
Day 4



Id1: B266B267IgMFITC II set.011
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	357.89	220.69	245.83

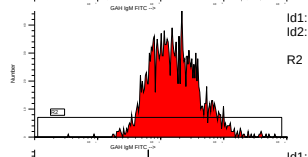
Day 7



Id1: B266B267IgMFITC II set.012
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	325.50	198.11	212.88

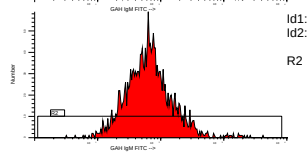
Day 14



Id1: B266B267IgMFITC II set.013
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	261.33	212.88	159.64

Day 21



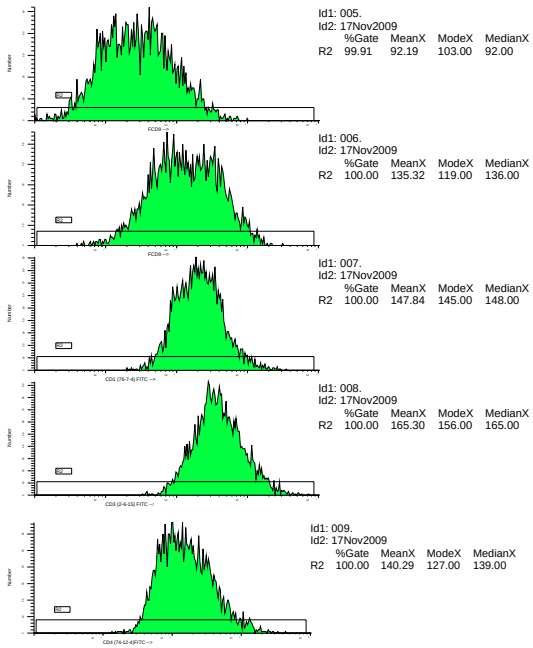
Id1: B266B267IgMFITC II set.014
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	102.46	62.65	64.94

Day 30

Anti-Gal IgG FACS

B266



Day 0

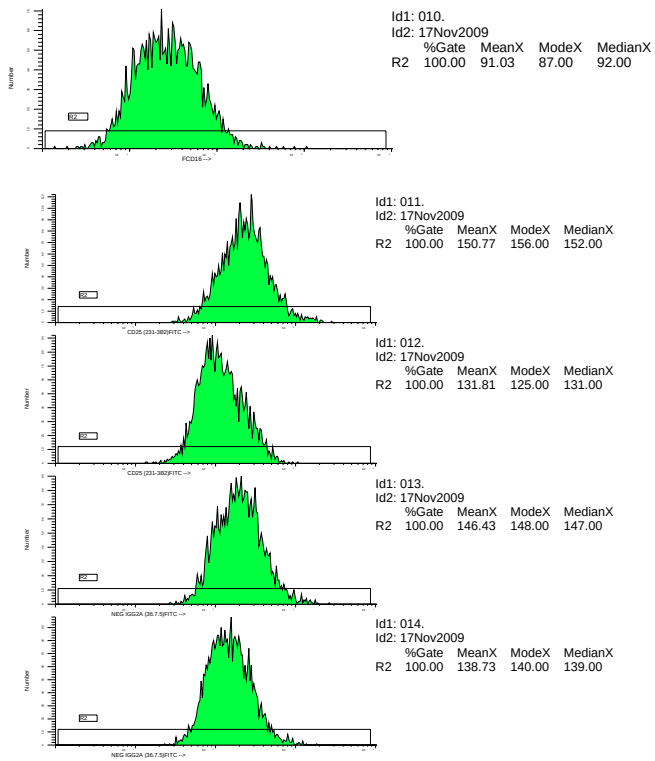
Day 7

Day 14

Day 21

Day 30

B267



Day 0

Day 7

Day 14

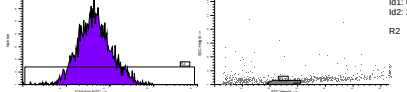
Day 21

Day 30

Anti-Gal IgG FACS Following Regrafting

GaH Fab IgM FITC (CON-642)

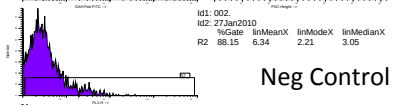
01/25/10 Opt Log:37042



Id1: 001.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	66.28	42.17	43.72

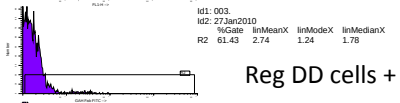
B268 POD14 (Pos Contr)



Id1: 002.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	88.15	6.34	2.21	3.05

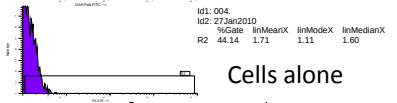
Neg Control



Id1: 003.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	61.43	2.74	1.24	1.78

Reg DD cells + II Ab

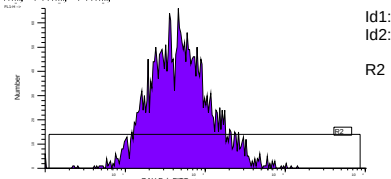


Id1: 004.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	44.14	1.71	1.11	1.60

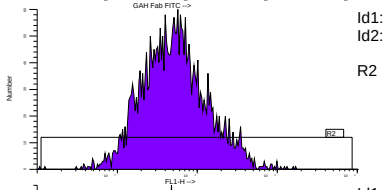
Cells alone

B266



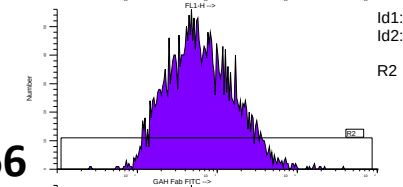
Id1: 005.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.81	74.65	45.32	48.70



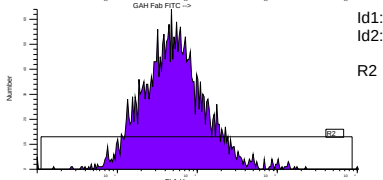
Id1: 006.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.97	82.80	56.23	52.33



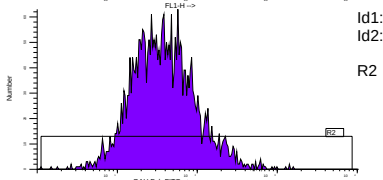
Id1: 007.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	102.24	46.98	60.43



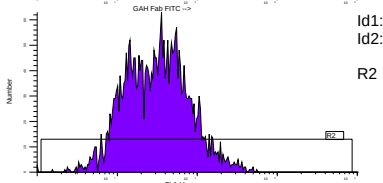
Id1: 008.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.80	83.41	46.98	50.48



Id1: 009.
Id2: 27Jan2010

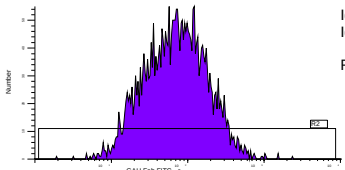
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	60.25	56.23	37.86



Id1: 010.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.90	47.35	35.23	32.78

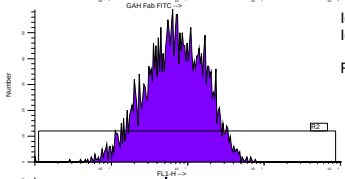
Day 0



Id1: 011.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	103.98	56.23	72.34

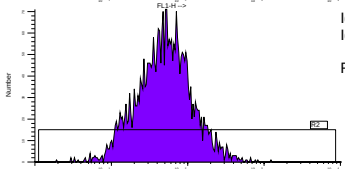
Day 4



Id1: 012.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	96.73	62.65	72.34

Day 7

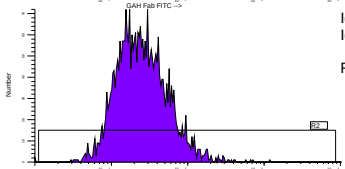


Id1: 013.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	69.18	50.48	52.33

B267

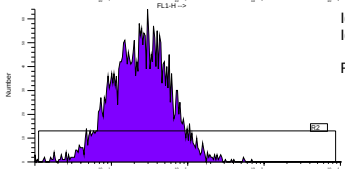
Day 14



Id1: 014.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	34.96	15.40	24.58

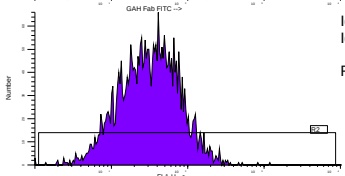
Day 21



Id1: 015.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.87	35.22	29.43	25.48

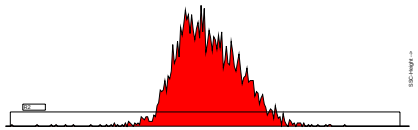
Day 30

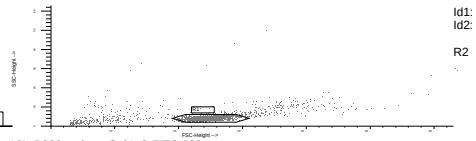


Id1: 016.
Id2: 27Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.91	45.57	40.68	32.78

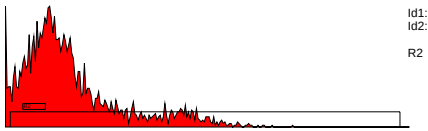
B267 POD 4 II set (Pos Contr)





Id1: B266 anti-nonGal IgG FITC.001
Id2: 6Jan2010

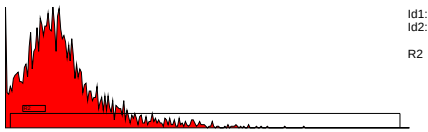
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	135.71	86.60	96.47



Id1: B266 anti-nonGal IgG FITC.002
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	89.97	12.71	2.74	3.52

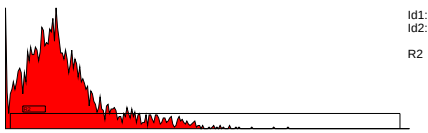
Neg Control



Id1: B266 anti-nonGal IgG FITC.003
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	90.49	8.40	2.94	3.40

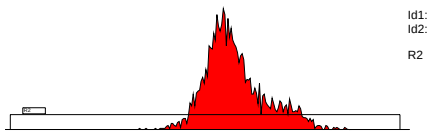
Gal KO cells + II Ab



Id1: B266 anti-nonGal IgG FITC.004
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	90.41	7.92	3.16	3.40

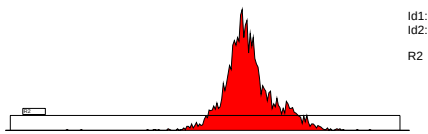
Cells alone



Id1: B266 anti-nonGal IgG FITC.005
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	249.89	143.31	171.55

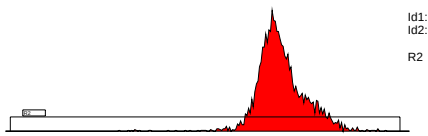
Day 0



Id1: B266 anti-nonGal IgG FITC.006
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	313.92	220.69	245.83

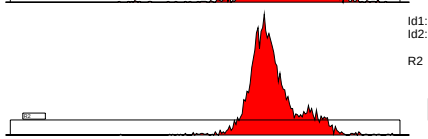
Day 4



Id1: B266 anti-nonGal IgG FITC.007
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	643.08	437.15	523.31

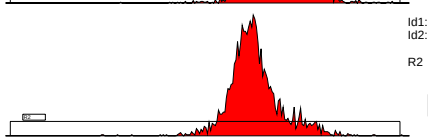
Day 7



Id1: B266 anti-nonGal IgG FITC.008
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	542.16	365.19	421.72

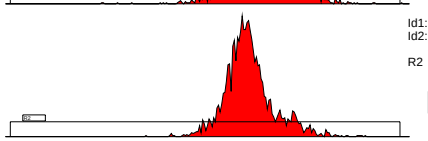
Day 14



Id1: B266 anti-nonGal IgG FITC.009
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	99.97	360.93	283.89	283.89

Day 21



Id1: B266 anti-nonGal IgG FITC.010
Id2: 6Jan2010

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	314.37	220.69	254.85

Day 30

Anti-nonGal IgG FACS Following Regrafting

GaH Fab IgM FITC (CON-642)

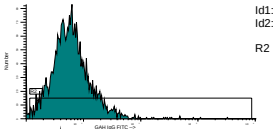
01/05/10 Opt Log: 336898

B266

Anti-nonGal IgG FACS Following Regrafting

GaH Fab IgM FITC (CON-642)

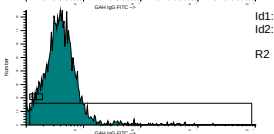
12/10/09 Opt Log: 36740



Id1: B266B267IgG FITC II set.015
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.44	10.70	6.26	6.04

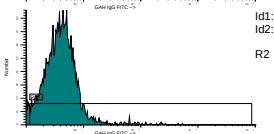
B268 POD14 (Pos Contr)



Id1: B266B267IgG FITC II set.016
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	95.18	8.09	3.92	4.07

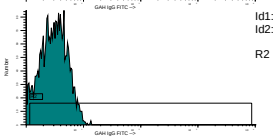
Neg Control



Id1: B266B267IgG FITC II set.017
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.11	6.37	4.37	4.07

Reg DD cells + II Ab

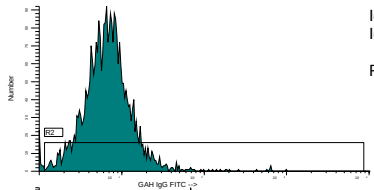


Id1: B266B267IgG FITC II set.018
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	90.99	3.57	4.53	3.28

Cells alone

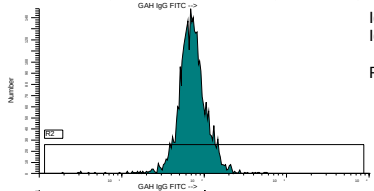
B267



Id1: B266B267IgG FITC II set.024
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.94	10.09	6.49	6.98

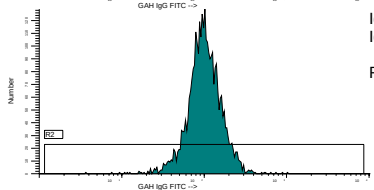
Day 0



Id1: B266B267IgG FITC II set.032
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	78.04	67.32	72.34

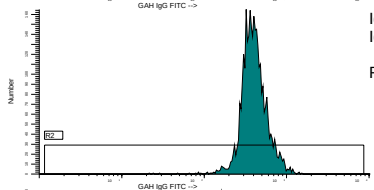
Day 4



Id1: B266B267IgG FITC II set.025
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	111.32	100.00	103.67

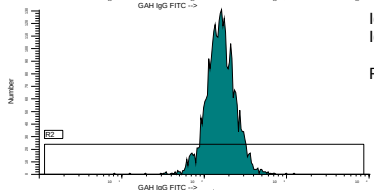
Day 7



Id1: B266B267IgG FITC II set.026
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	423.86	316.23	406.80

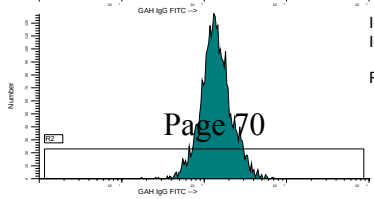
Day 14



Id1: B266B267IgG FITC II set.027
Id2: 10Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	171.51	159.64	159.64

Day 21



Id1: B266B267IgG FITC II set.028
Id2: 10Dec2009

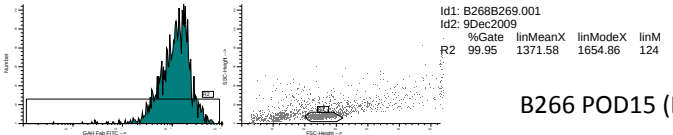
	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	152.27	128.64	138.24

Day 30

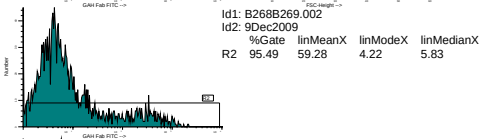
Anti-Gal IgM FACS

GAH Fab IgM FITC (CON-635)

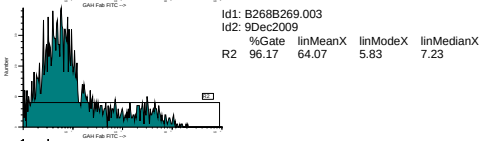
12/09/09 Opt Log: 36727



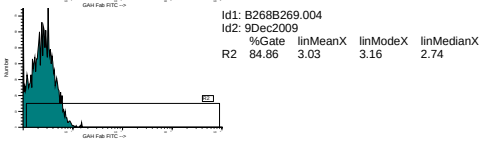
B266 POD15 (Pos Contr)



Neg Control

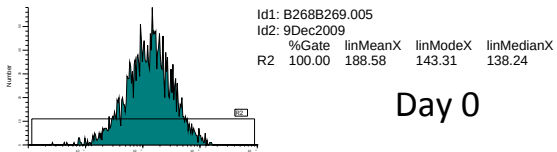


Reg DD cells + II Ab

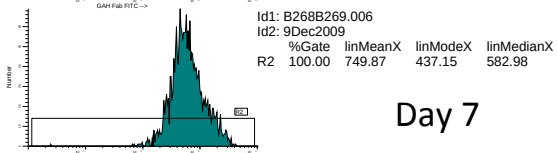


Cells alone

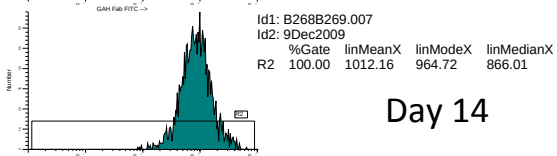
B268



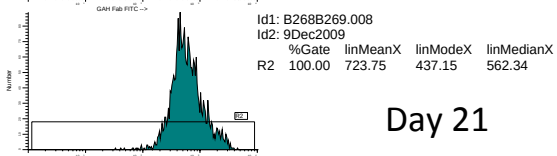
Day 0



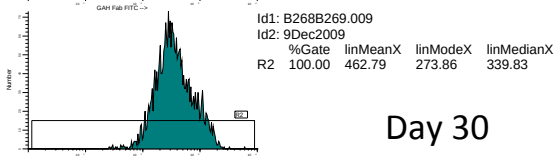
Day 7



Day 14

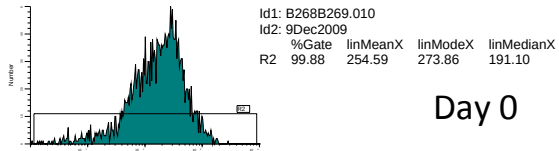


Day 21

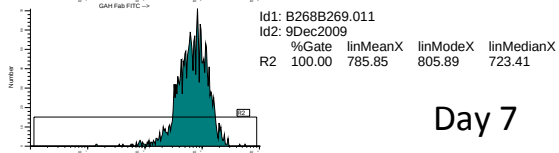


Day 30

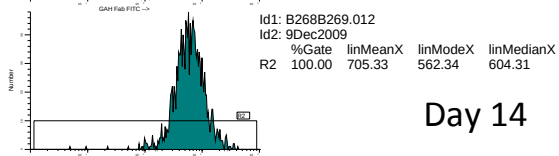
B269



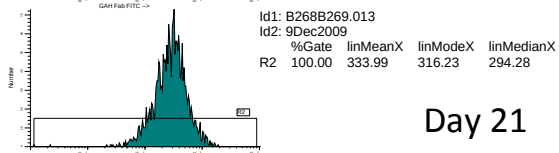
Day 0



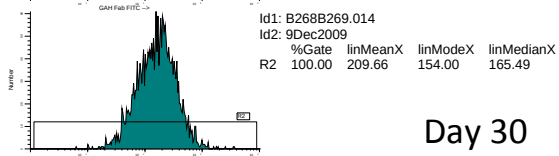
Day 7



Day 14



Day 21

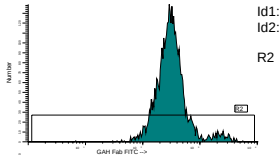


Day 30

Anti-nonGal IgM FACS

GAH Fab IgM FITC (CON-635)

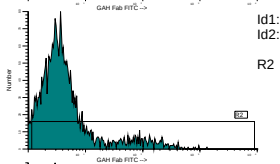
12/09/09 Opt Log: 36727



Id1: B268B269.015
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	411.05	283.89	305.07

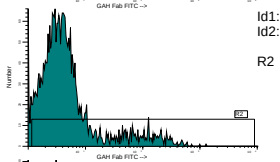
B266 POD15 (Pos Contr)



Id1: B268B269.016
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	91.21	18.85	3.65	3.79

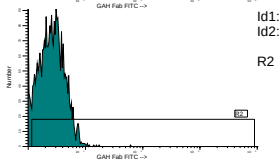
Neg Control



Id1: B268B269.017
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	91.75	21.05	2.94	4.07

Reg KO cells + II Ab

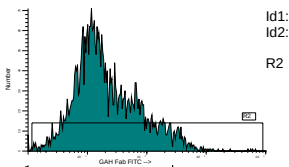


Id1: B268B269.018
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	87.43	3.04	3.05	2.74

Cells alone

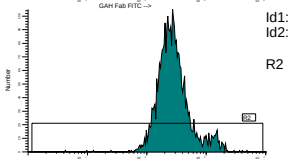
B268



Id1: B268B269.019
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.76	60.60	11.55	17.16

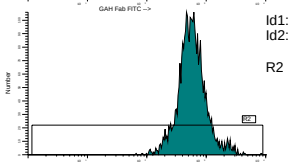
Day 0



Id1: B268B269.020
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	355.23	264.18	264.18

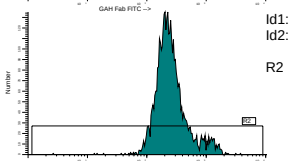
Day 7



Id1: B268B269.021
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	670.53	453.19	562.34

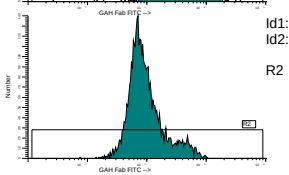
Day 14



Id1: B268B269.022
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	318.04	198.11	237.15

Day 21

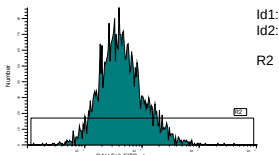


Id1: B268B269.023
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	127.31	69.79	83.5

Day 30

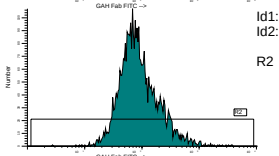
B269



Id1: B268B269.024
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	69.65	39.24	42.17

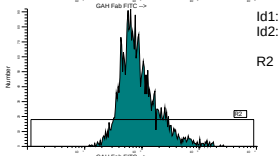
Day 0



Id1: B268B269.025
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	128.73	67.32	80.59

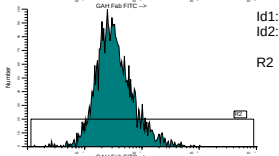
Day 7



Id1: B268B269.026
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	128.89	62.65	77.74

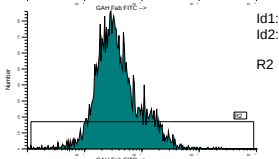
Day 14



Id1: B268B269.027
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	46.26	24.58	31.62

Day 21



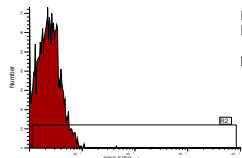
Id1: B268B269.028
Id2: 9Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.95	60.59	28.39	33.98

Day 30

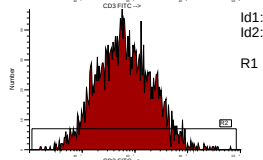
Anti-Gal IgG FACS

B268



Id1: 004.
Id2: 17Nov2009

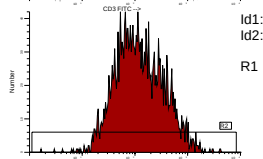
	%Gate	MeanX	ModeX	Med
R1	52.76	24.49	0.00	0.00



Id1: 005.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	61.48	24.26	0.00	0.00

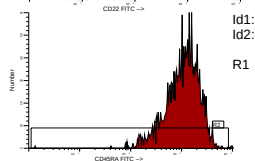
Day 0



Id1: 006.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	54.49	24.01	0.00	0.00

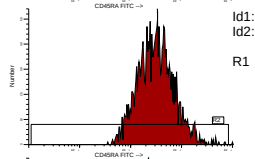
Day 7



Id1: 007.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	45.17	24.04	0.00	0.00

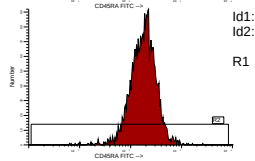
Day 14



Id1: 008.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	45.80	24.07	0.00	0.00

Day 21

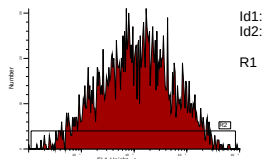


Id1: 009.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	59.36	24.45	0.00	0.00

Day 30

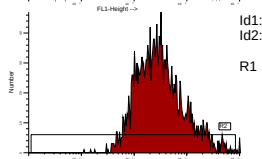
B269



Id1: 010.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	55.77	24.46	0.00	0.00

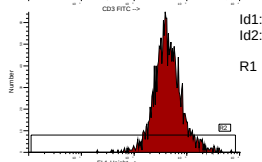
Day 0



Id1: 011.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	46.48	23.90	0.00	0.00

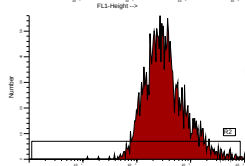
Day 7



Id1: 012.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	40.04	23.87	0.00	0.00

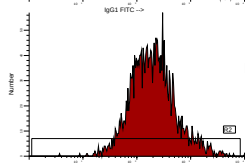
Day 14



Id1: 013.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	50.58	24.15	0.00	0.00

Day 21



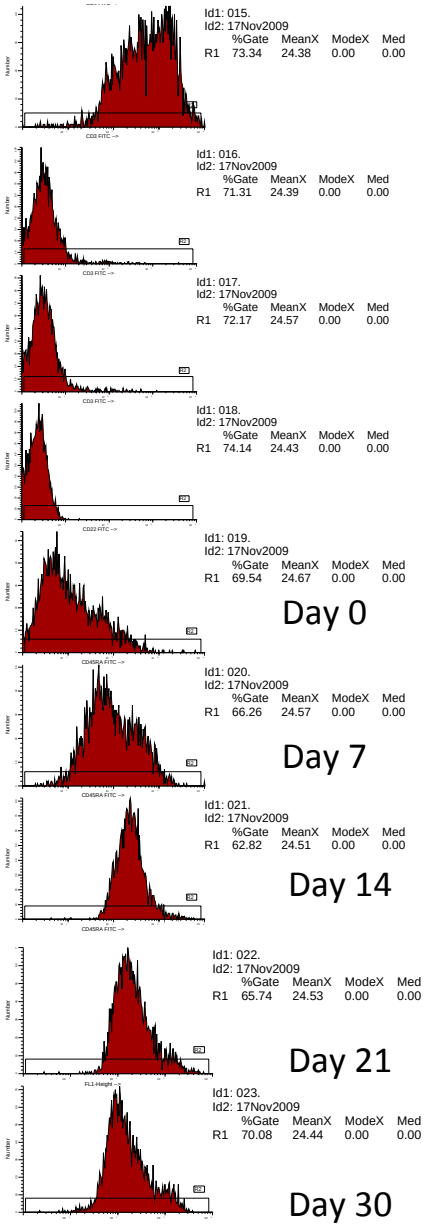
Id1: 014.
Id2: 17Nov2009

	%Gate	MeanX	ModeX	Med
R1	53.29	24.31	0.00	0.00

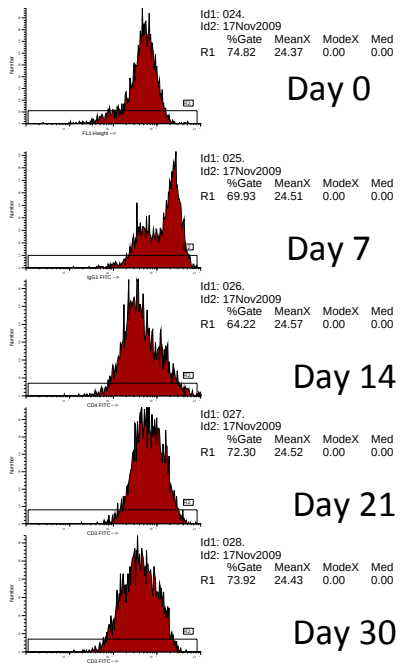
Day 30

Anti-nonGal IgG

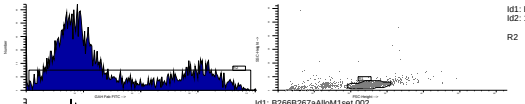
B268



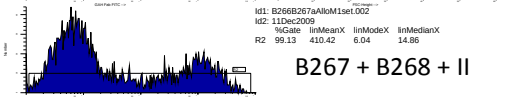
B269



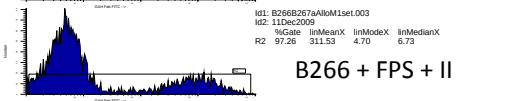
B266-B267 Anti-Allo Facs
GaH IgM Fab FITC Ab
12/11/2009
Opt log 36751



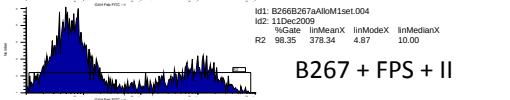
B266 + B268 + II



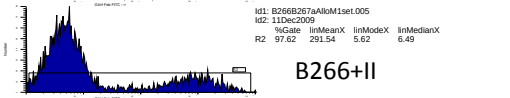
B267 + B268 + II



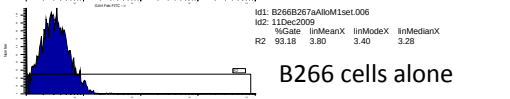
B266 + FPS + II



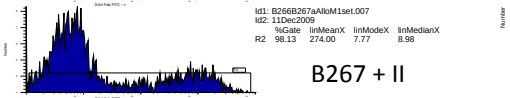
B267 + FPS + II



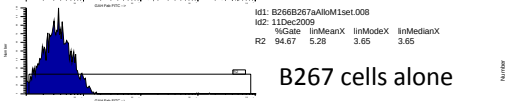
B266+II



B266 cells alone

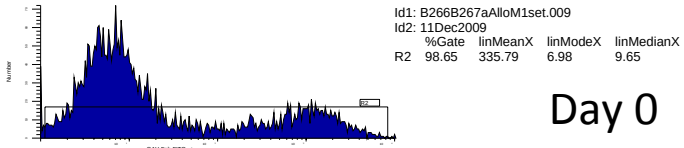


B267 + II

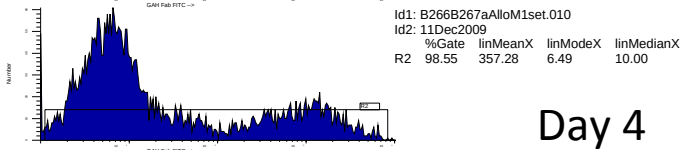


B267 cells alone

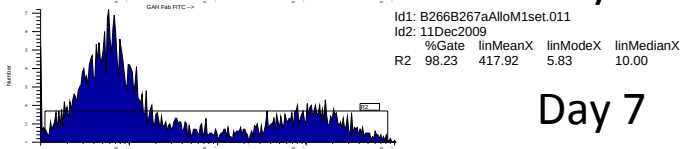
B267



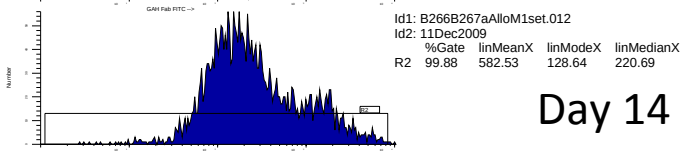
Day 0



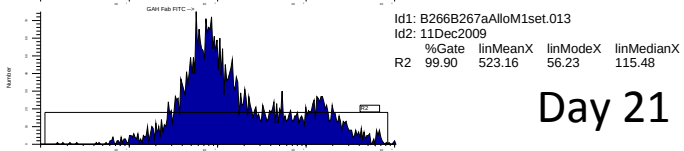
Day 4



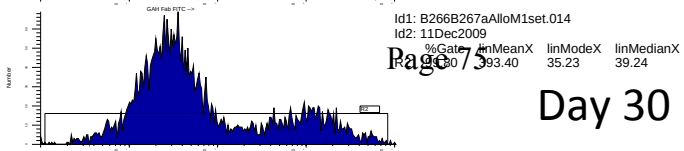
Day 7



Day 14

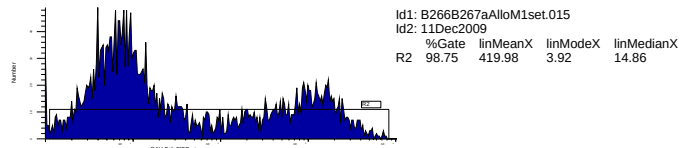


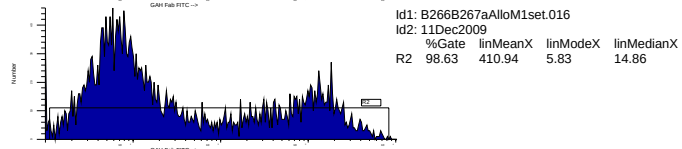
Day 21

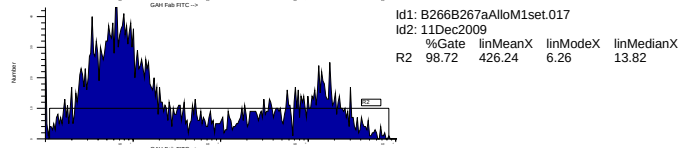


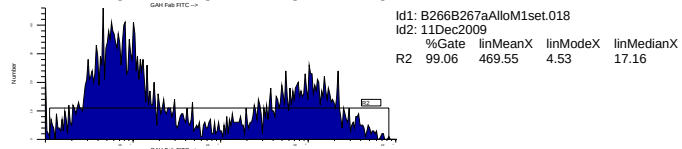
Day 30

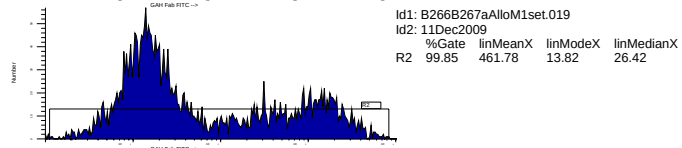
B266

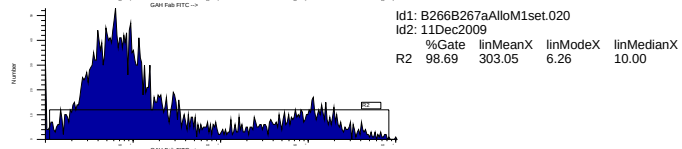








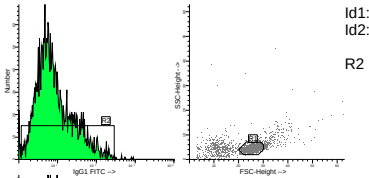




Anti-Allo IgG Fab Facs

11/28/2009

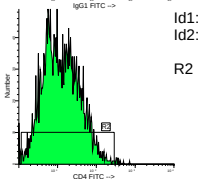
Opt log 36650



Id1: 001.
Id2: 2Dec2009

	%Gate	linMeanX
R2	98.52	17.58

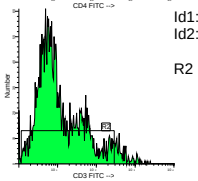
B266+B158+II



Id1: 002.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.03	21.33	5.62	11.97

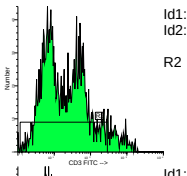
B267+B158+II



Id1: 003.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.05	25.69	4.87	7.77

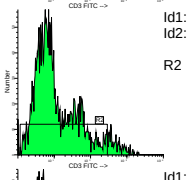
B266+FPS+II



Id1: 004.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	91.89	37.30	6.49	15.96

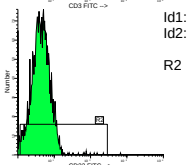
B267+FPS+II



Id1: 005.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	93.82	24.43	5.62	7.77

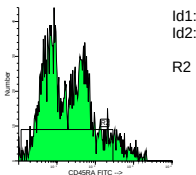
B266+II



Id1: 006.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	96.87	5.32	5.05	4.53

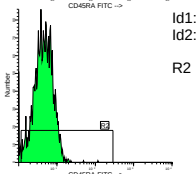
B266 cells alone



Id1: 007.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	92.28	35.61	8.35	15.40

B267+II

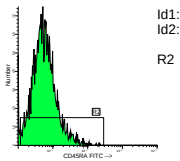


Id1: 008.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.49	5.22	3.79	4.70

B267 cells alone

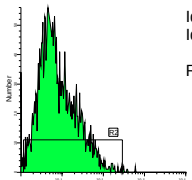
B267



Id1: 009.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.60	8.30	5.05	5.43

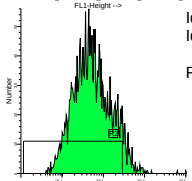
Day 0



Id1: 010.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.32	14.42	4.53	6.73

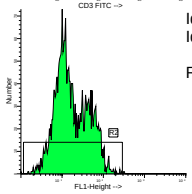
Day 7



Id1: 011.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	93.34	69.40	43.72	48.70

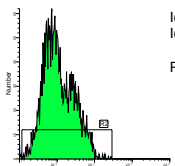
Day 14



Id1: 012.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.90	26.65	10.00	14.86

Day 21

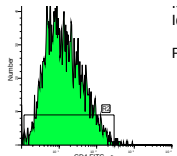


Id1: 013.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.64	17.47	7.23	10.00

Day 30

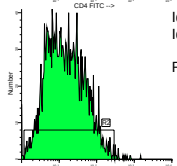
B266



Id1: 014.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.87	24.42	4.70	12.41

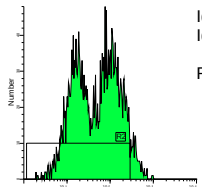
Day 0



Id1: 015.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.74	27.46	6.49	13.82

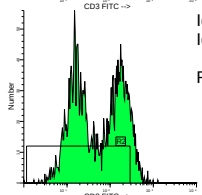
Day 7



Id1: 016.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.78	72.32	74.99	48.70

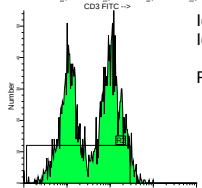
Day 14



Id1: 017.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	91.30	80.32	14.86	39.24

Day 21



Id1: 018.
Id2: 2Dec2009

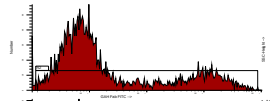
	%Gate	linMeanX	linModeX	linMedianX
R2	97.01	69.30	119.71	50.48

Day 30

B266-B267 Anti-Allo Facs
Following II set of Grafts
GaH IgM Fab FITC Ab

12/11/2009

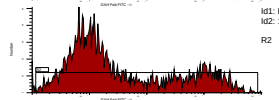
Opt log 36752



Id1: B266B267aAlloM2set.001
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.49	331.45	9.65	10.00

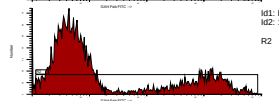
B266 + B268 + II



Id1: B266B267aAlloM2set.002
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.08	348.16	6.49	16.55

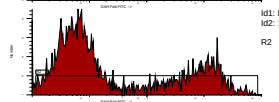
B267 + B268 + II



Id1: B266B267aAlloM2set.003
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.47	369.79	4.37	6.49

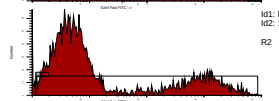
B266 + FPS + II



Id1: B266B267aAlloM2set.004
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.21	385.24	6.26	10.37

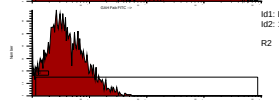
B267 + FPS + II



Id1: B266B267aAlloM2set.005
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	96.69	325.53	3.65	6.49

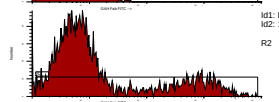
B266+II



Id1: B266B267aAlloM2set.006
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	93.89	5.64	2.64	3.79

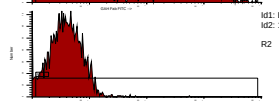
B266 cells alone



Id1: B266B267aAlloM2set.007
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.98	241.86	4.87	8.06

B267 + II

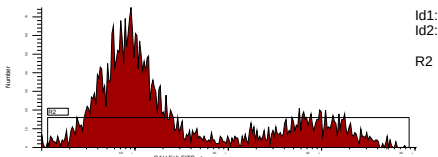


Id1: B266B267aAlloM2set.008
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.47	5.03	3.28	4.07

B267 cells alone

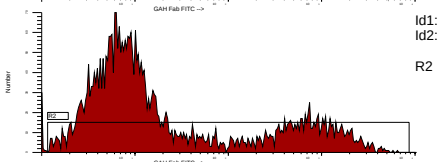
B267



Id1: B266B267aAlloM2set.009
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.76	288.95	8.98	11.55

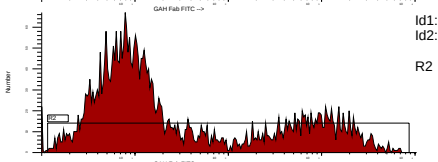
Day 0



Id1: B266B267aAlloM2set.010
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.12	253.40	6.04	10.37

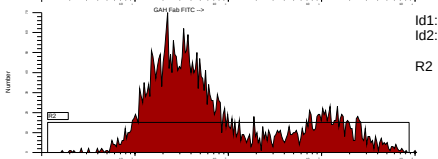
Day 4



Id1: B266B267aAlloM2set.011
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.37	336.67	7.77	12.86

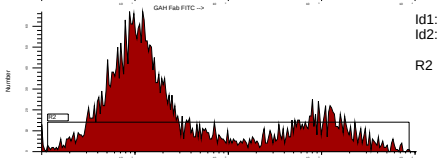
Day 7



Id1: B266B267aAlloM2set.012
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.97	420.11	22.07	43.72

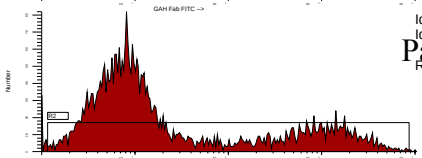
Day 14



Id1: B266B267aAlloM2set.013
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.53	339.26	8.66	14.86

Day 21

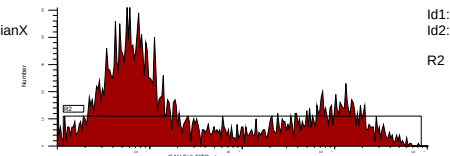


Id1: B266B267aAlloM2set.014
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.73	350.67	8.06	10.75

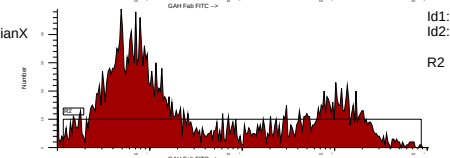
Day 30

B266



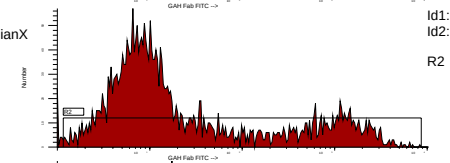
Id1: B266B267aAlloM2set.015
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.05	374.97	5.62	11.55



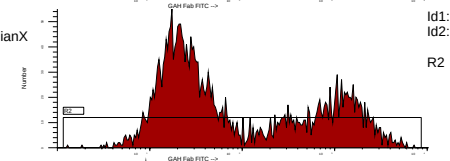
Id1: B266B267aAlloM2set.016
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.25	378.80	4.87	12.86



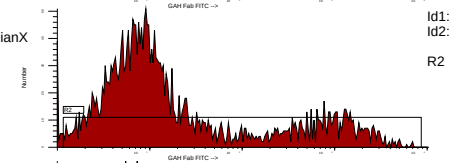
Id1: B266B267aAlloM2set.017
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.92	291.28	6.49	11.97



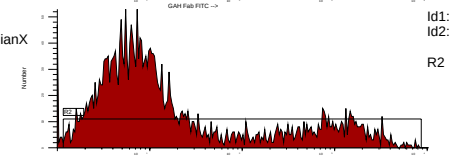
Id1: B266B267aAlloM2set.018
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	463.40	17.16	42.17



Id1: B266B267aAlloM2set.019
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.26	259.13	8.98	10.75



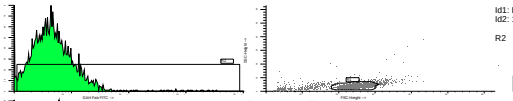
Id1: B266B267aAlloM2set.020
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.10	284.10	5.43	9.65

B266-B267 Anti-Allo Facs Following II set of Grafts GaH IgG Fab FITC Ab

12/11/2009

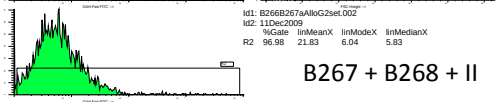
Opt log 36753



Id1: B266B267aAlloG2set.001
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	95.20	16.11	4.37	4.53

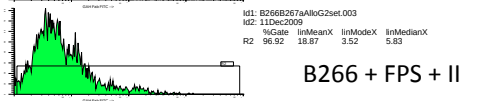
B266 + B268 + II



Id1: B266B267aAlloG2set.002
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	96.98	21.83	6.04	5.83

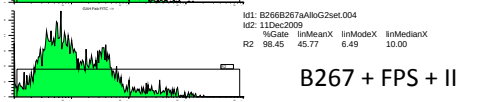
B267 + B268 + II



Id1: B266B267aAlloG2set.003
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	96.92	18.87	3.52	5.83

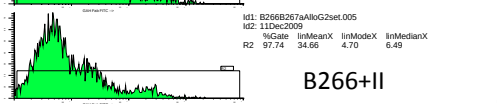
B266 + FPS + II



Id1: B266B267aAlloG2set.004
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.45	45.77	6.49	10.00

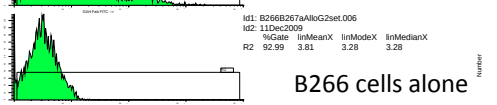
B267 + FPS + II



Id1: B266B267aAlloG2set.005
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.74	34.66	4.70	6.49

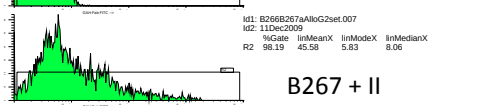
B266+II



Id1: B266B267aAlloG2set.006
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	92.99	3.81	3.28	3.28

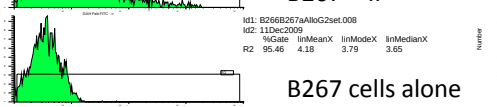
B266 cells alone



Id1: B266B267aAlloG2set.007
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.19	45.58	5.83	8.06

B267 + II



Id1: B266B267aAlloG2set.008
Id2: 11Dec2009

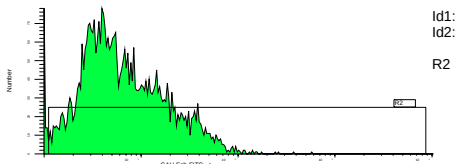
	%Gate	linMeanX	linModeX	linMedianX
R2	95.46	4.18	3.79	3.65

B267 cells alone

B267

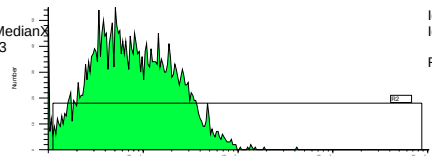
B266

Day 0



Id1: B266B267aAlloG2set.009
Id2: 11Dec2009

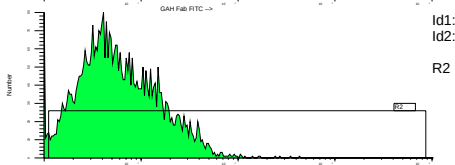
	%Gate	linMeanX	linModeX	linMedianX
R2	97.22	11.77	3.92	5.83



Id1: B266B267aAlloG2set.015
Id2: 11Dec2009

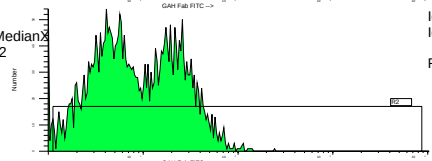
	%Gate	linMeanX	linModeX	linMedianX
R2	97.52	13.06	5.05	7.77

Day 4



Id1: B266B267aAlloG2set.010
Id2: 11Dec2009

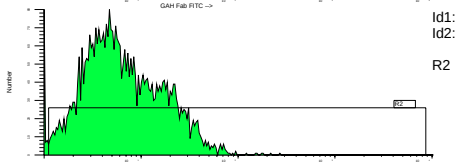
	%Gate	linMeanX	linModeX	linMedianX
R2	96.40	9.89	4.07	5.62



Id1: B266B267aAlloG2set.016
Id2: 11Dec2009

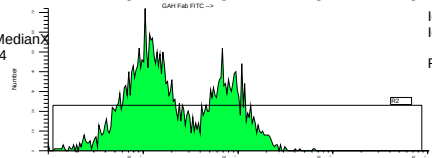
	%Gate	linMeanX	linModeX	linMedianX
R2	97.55	14.47	4.07	8.35

Day 7



Id1: B266B267aAlloG2set.011
Id2: 11Dec2009

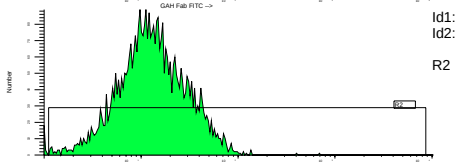
	%Gate	linMeanX	linModeX	linMedianX
R2	97.32	10.52	4.70	6.04



Id1: B266B267aAlloG2set.017
Id2: 11Dec2009

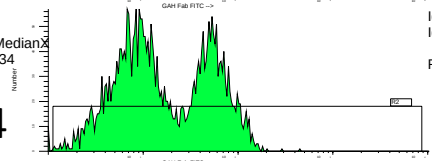
	%Gate	linMeanX	linModeX	linMedianX
R2	99.91	41.84	10.37	17.78

Day 14



Id1: B266B267aAlloG2set.012
Id2: 11Dec2009

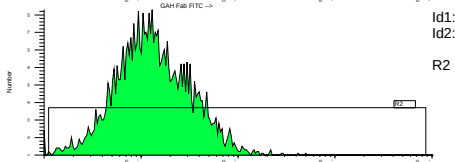
	%Gate	linMeanX	linModeX	linMedianX
R2	99.45	17.96	9.65	13.34



Id1: B266B267aAlloG2set.018
Id2: 11Dec2009

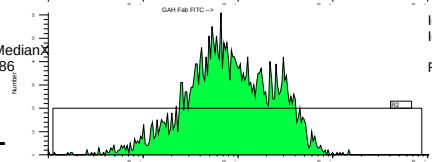
	%Gate	linMeanX	linModeX	linMedianX
R2	99.69	30.02	6.73	13.82

Day 21



Id1: B266B267aAlloG2set.013
Id2: 11Dec2009

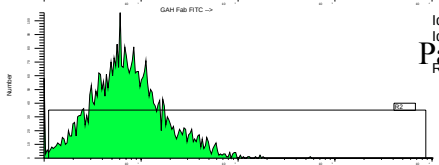
	%Gate	linMeanX	linModeX	linMedianX
R2	99.65	19.78	12.86	12.86



Id1: B266B267aAlloG2set.019
Id2: 11Dec2009

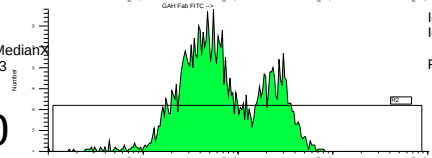
	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	133.91	64.94	80.59

Day 30



Id1: B266B267aAlloG2set.014
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.32	11.27	6.04	7.23

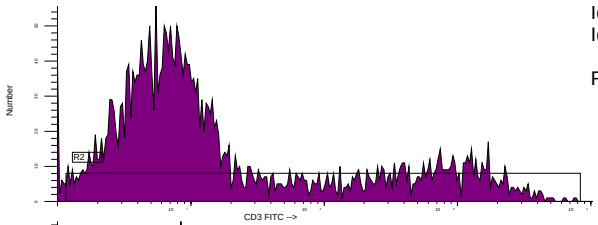


Id1: B266B267aAlloG2set.020
Id2: 11Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	112.87	54.25	60.43

Anti-Allo IgM Facs

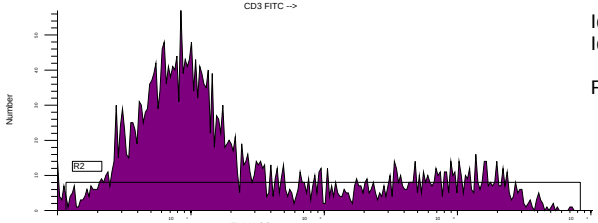
B268



Id1: gahIgMFabB268B269cont.002
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.35	238.58	5.43	9.31

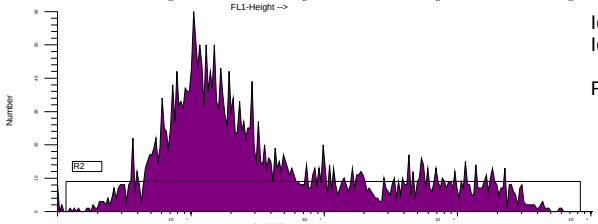
POD 0



Id1: gahIgMFabB268B269cont.003
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.11	261.34	8.35	12.41

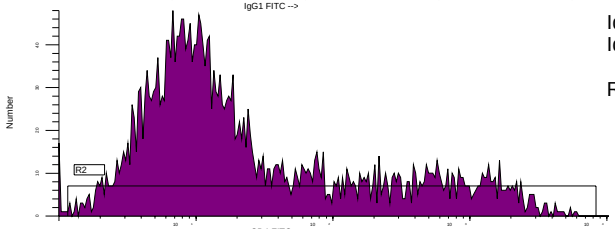
POD 7



Id1: gahIgMFabB268B269cont.004
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.67	256.32	10.37	20.54

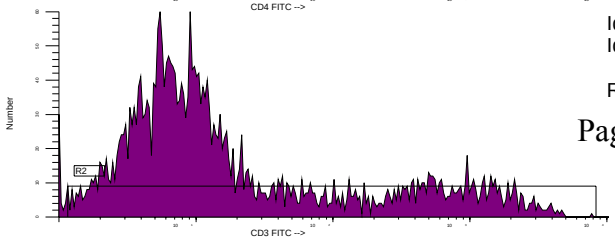
POD 15



Id1: gahIgMFabB268B269cont.005
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.35	215.96	6.73	13.82

POD 21



Id1: gahIgMFabB268B269cont.006
Id2: 8Dec2009

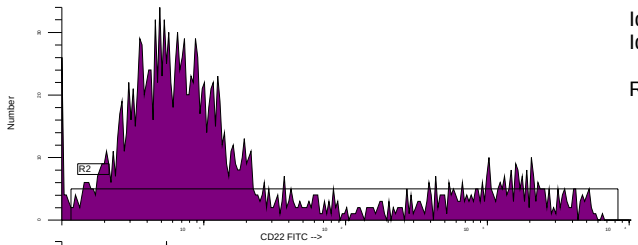
	%Gate	linMeanX	linModeX	linMedianX
R2	98.73	223.72	5.43	10.37

POD 30

B269

Anti-Allo Facs using GaH IgM Fab FITC

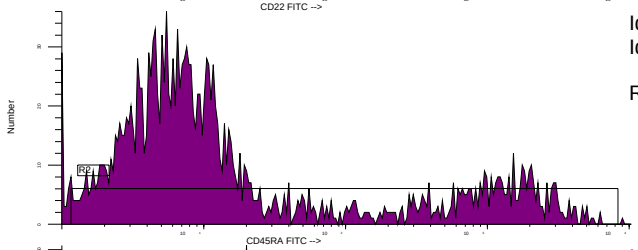
12/08/2009 Opt log: 36722



Id1: gahIgMFabB268B269.009
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.00	336.87	4.87	8.66

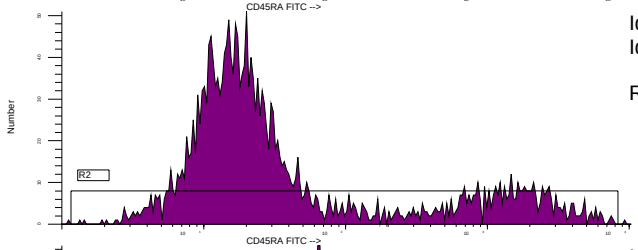
POD 0



Id1: gahIgMFabB268B269.010
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	97.81	312.72	5.43	8.66

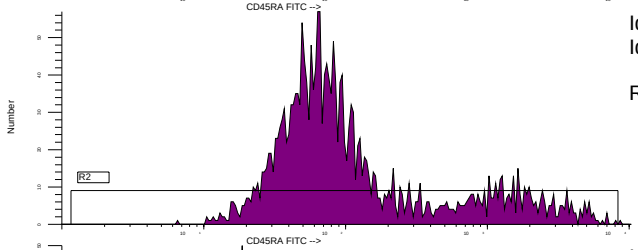
POD 7



Id1: gahIgMFabB268B269.011
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.91	372.40	19.81	20.54

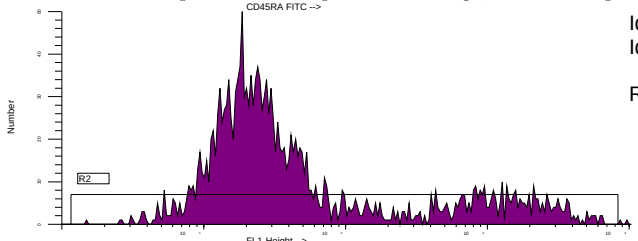
POD 15



Id1: gahIgMFabB268B269.012
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.95	431.43	62.65	77.74

POD 21



Id1: gahIgMFabB268B269cont.001
Id2: 8Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.89	346.81	18.44	27.39

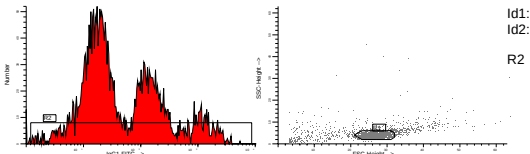
POD 30

Anti-Allo IgG Fab Facs

12/01/2009

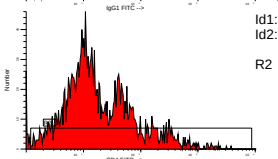
Opt log 36668

B268+B158+II



Id1: 001.
Id2: 2Dec2009

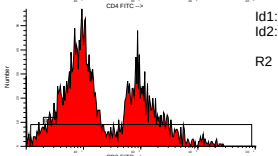
	%Gate	linMeanX	linModeX	linM
R2	99.48	207.59	17.16	28.3



Id1: 002.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.09	71.06	10.75	14.86

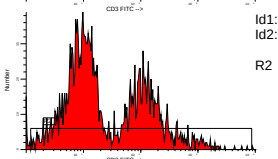
B269+158+II



Id1: 003.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.72	86.72	9.31	13.82

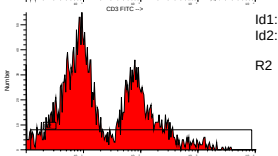
B268+FPS+II



Id1: 004.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.92	106.78	9.65	15.40

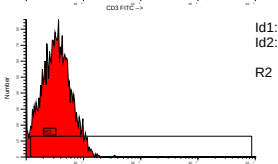
B269+FPS+II



Id1: 005.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.29	87.55	9.31	13.34

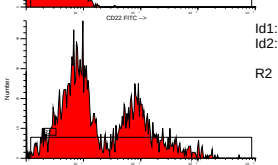
B268+II



Id1: 006.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	92.85	4.10	3.65	3.52

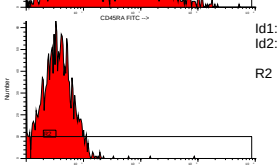
B268 cells alone



Id1: 007.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.50	81.53	9.65	11.97

B269+II

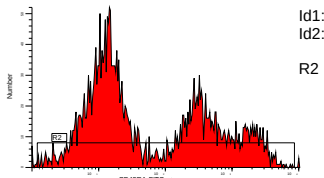


Id1: 008.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	94.01	4.78	3.28	3.65

B269 cells alone

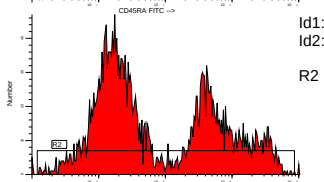
B269



Id1: 009.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.58	398.71	14.33	25.48

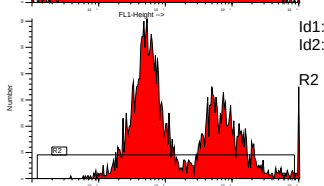
Day 0



Id1: 010.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.64	483.68	17.16	36.52

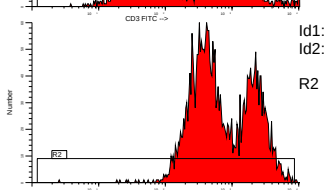
Day 7



Id1: 011.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	98.69	460.27	52.33	86.60

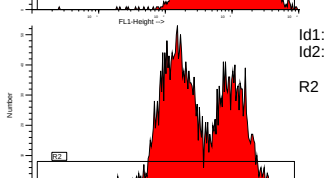
Day 14



Id1: 012.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.94	1171.73	316.23	582.98

Day 21

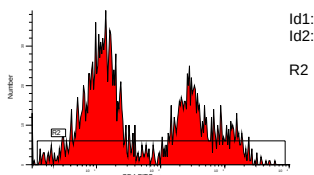


Id1: 013.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	100.00	562.13	148.56	264.18

Day 30

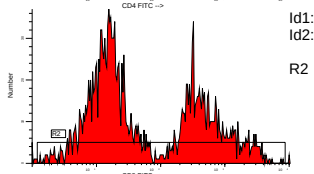
B268



Id1: 014.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.36	264.10	13.82	22.07

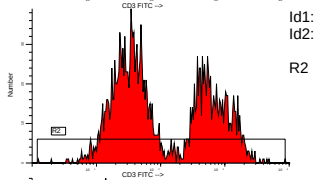
Day 0



Id1: 015.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.54	343.51	15.40	27.39

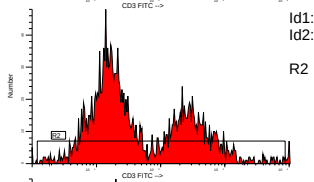
Day 7



Id1: 016.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.95	356.04	33.98	60.43

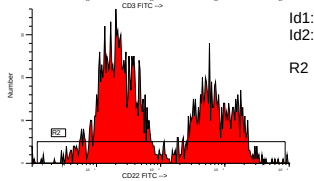
Day 14



Id1: 017.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.15	219.96	13.82	26.42

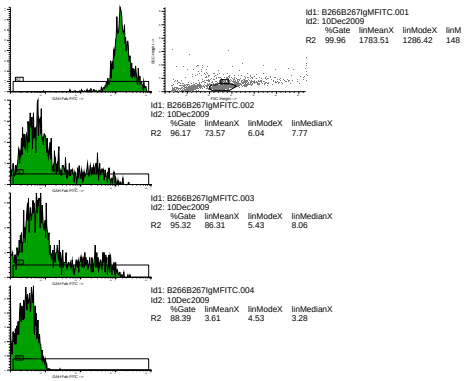
Day 21



Id1: 018.
Id2: 2Dec2009

	%Gate	linMeanX	linModeX	linMedianX
R2	99.68	384.21	19.81	42.17

Day 30

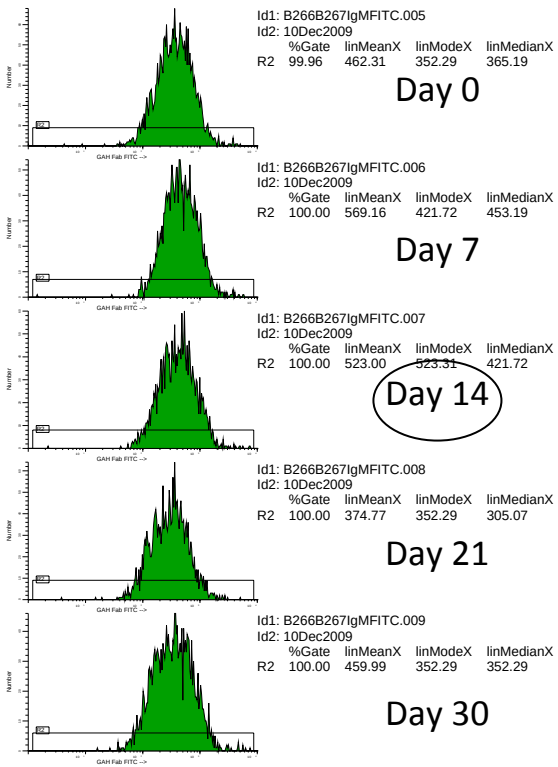


Does Re-grafting Accelerate Humoral Response?

Anti-Gal IgM FACS

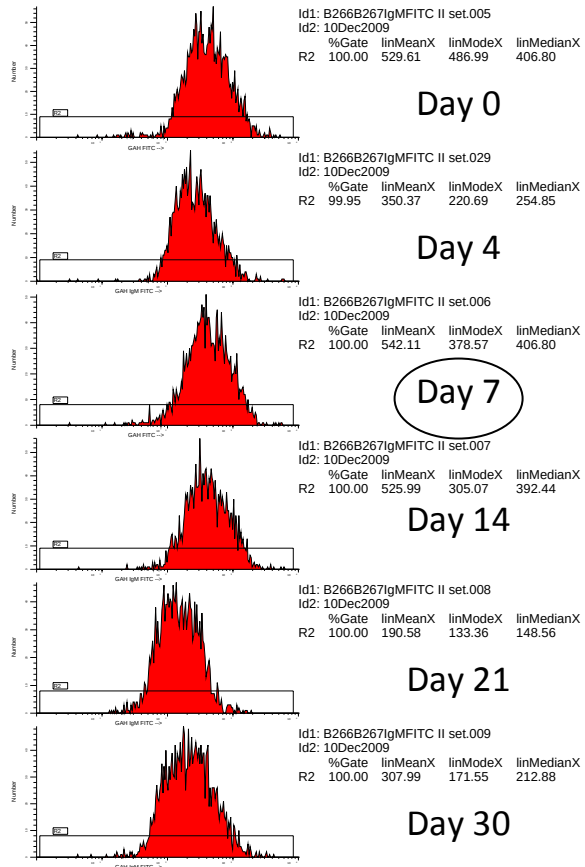
I set of Skin Grafts

B266

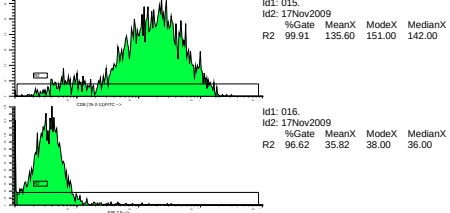


II set of Skin Grafts

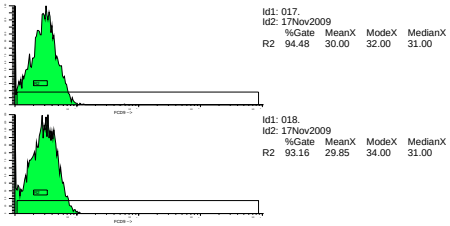
B266



Does Re-grafting Accelerate Humoral Response?



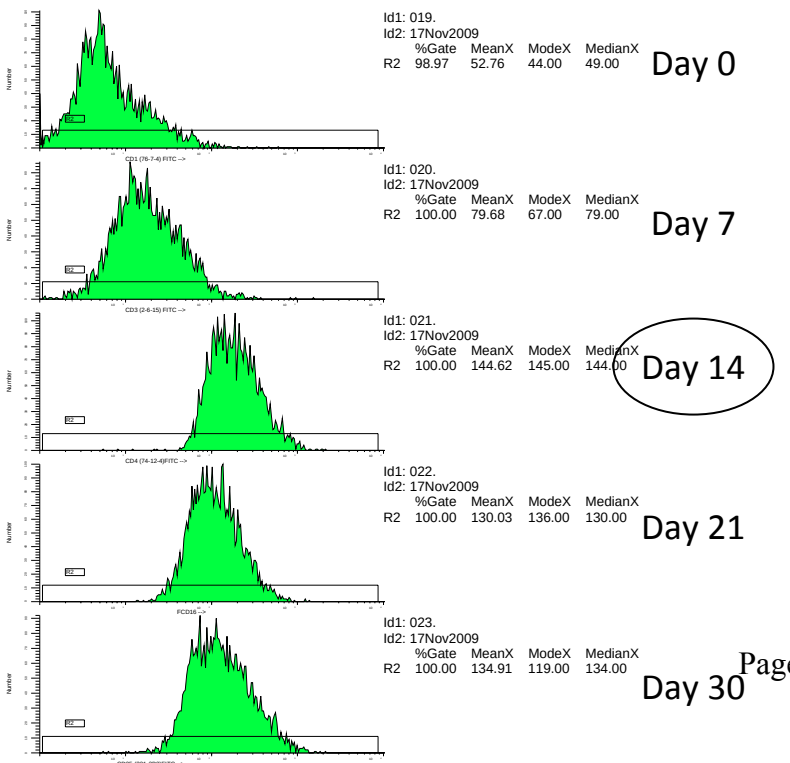
Anti-nonGal IgG FACS

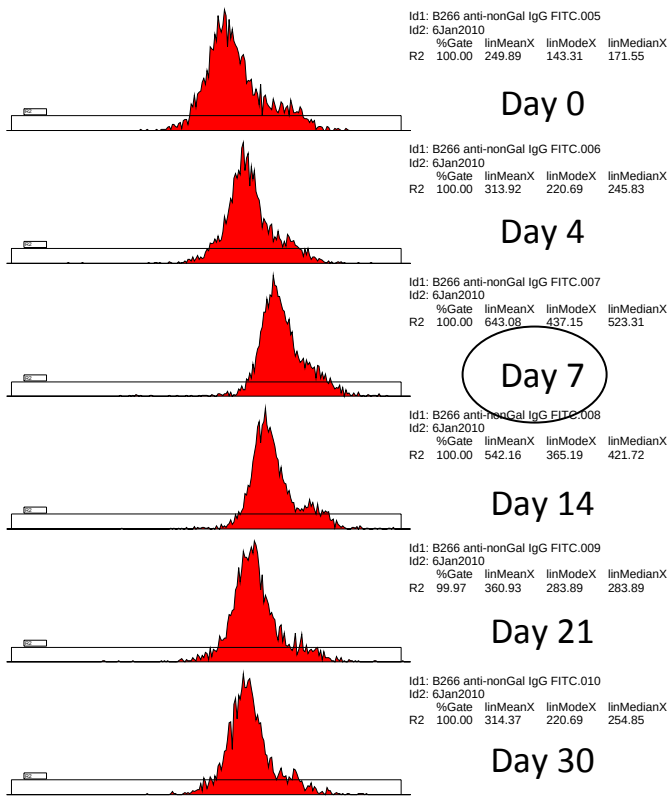


I set of Skin Grafts

II set of Skin Grafts

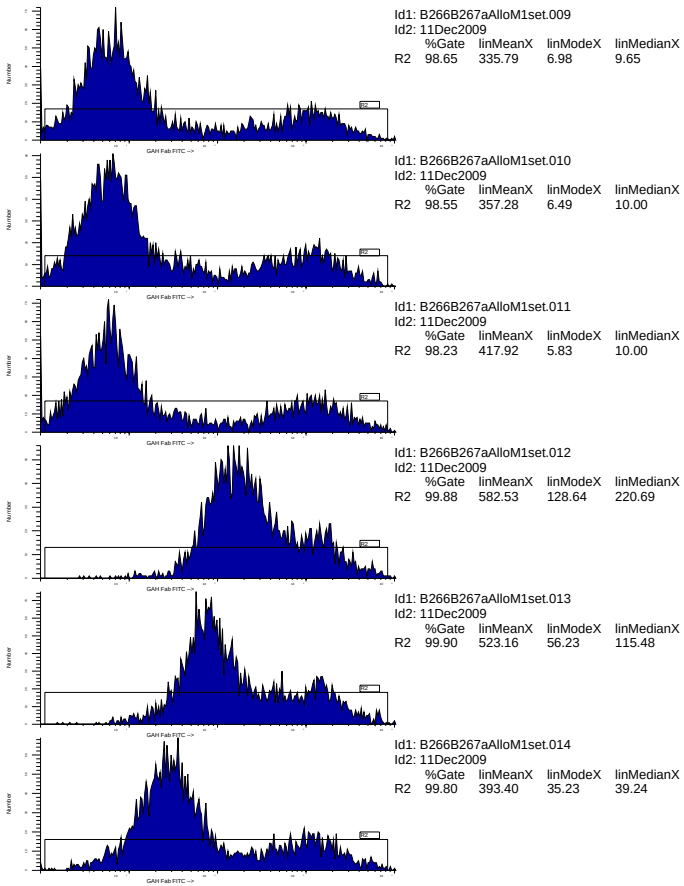
B266





B267 Anti-Allo IgM Facs

I set of skin grafts



Day 0

Day 4

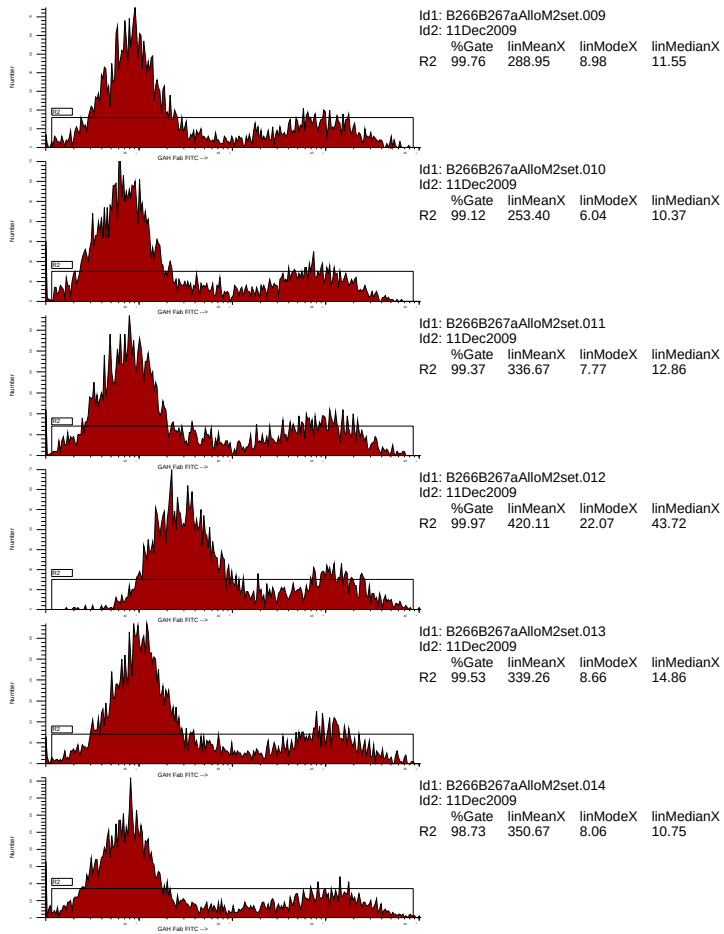
Day 7

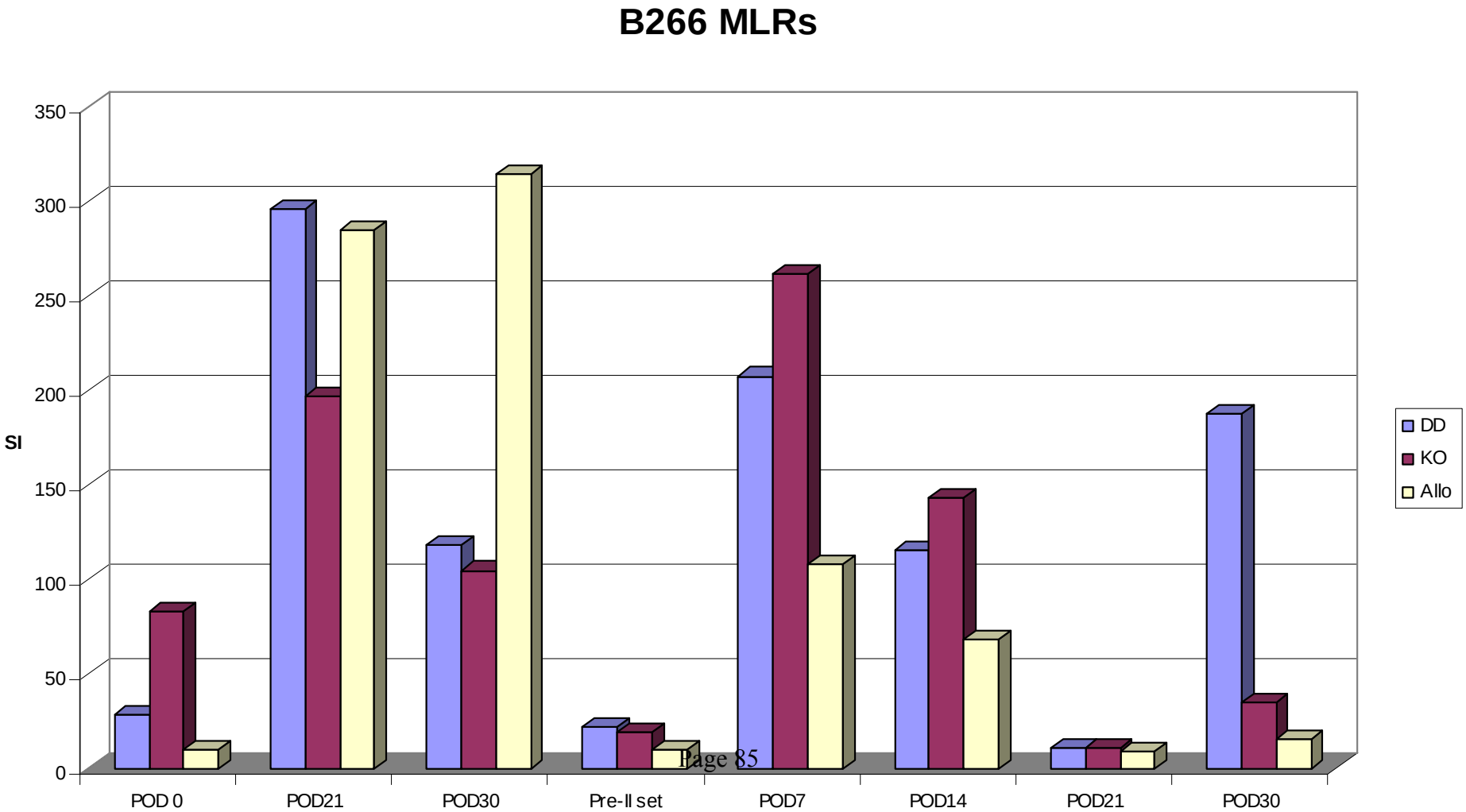
Day 14

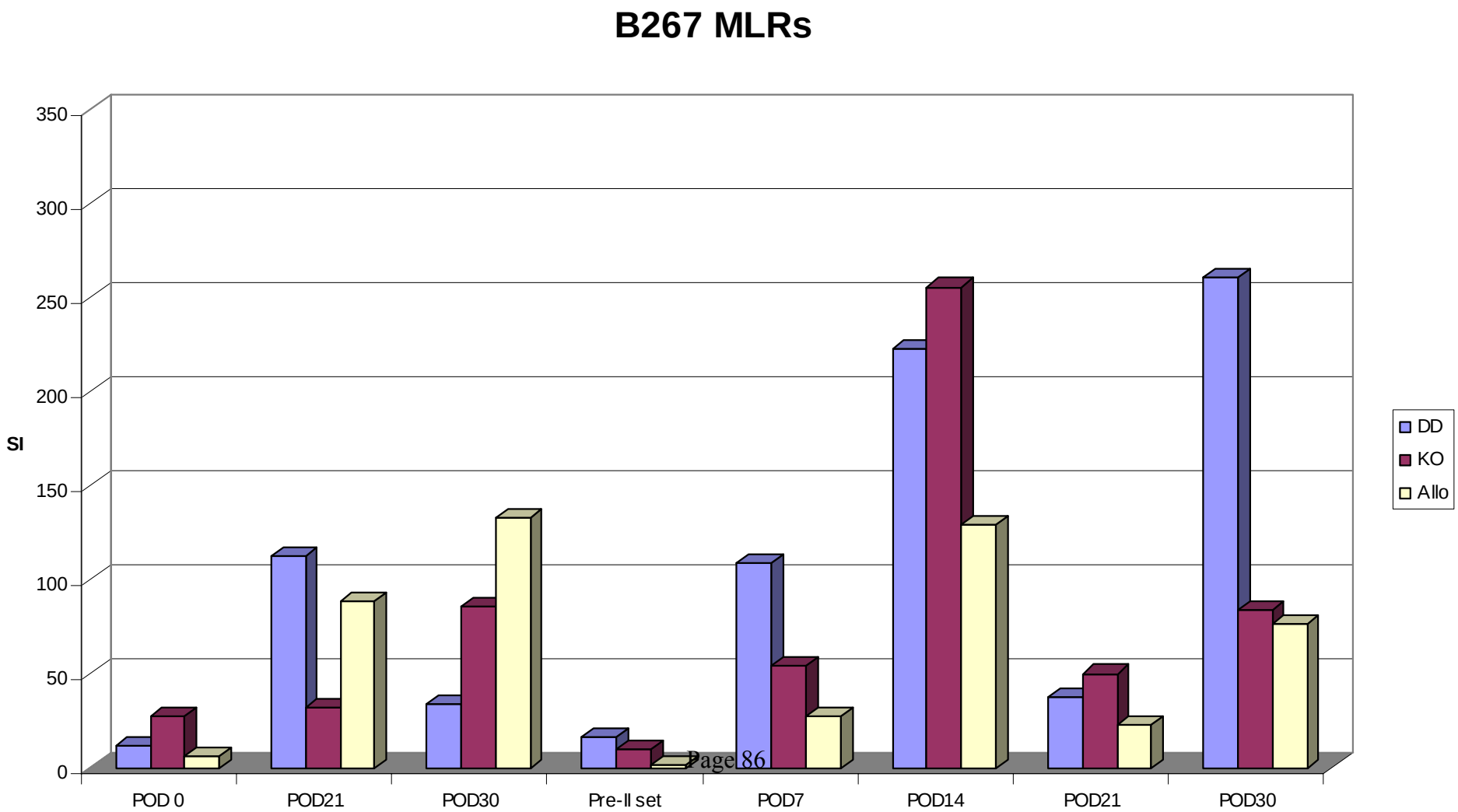
Day 21

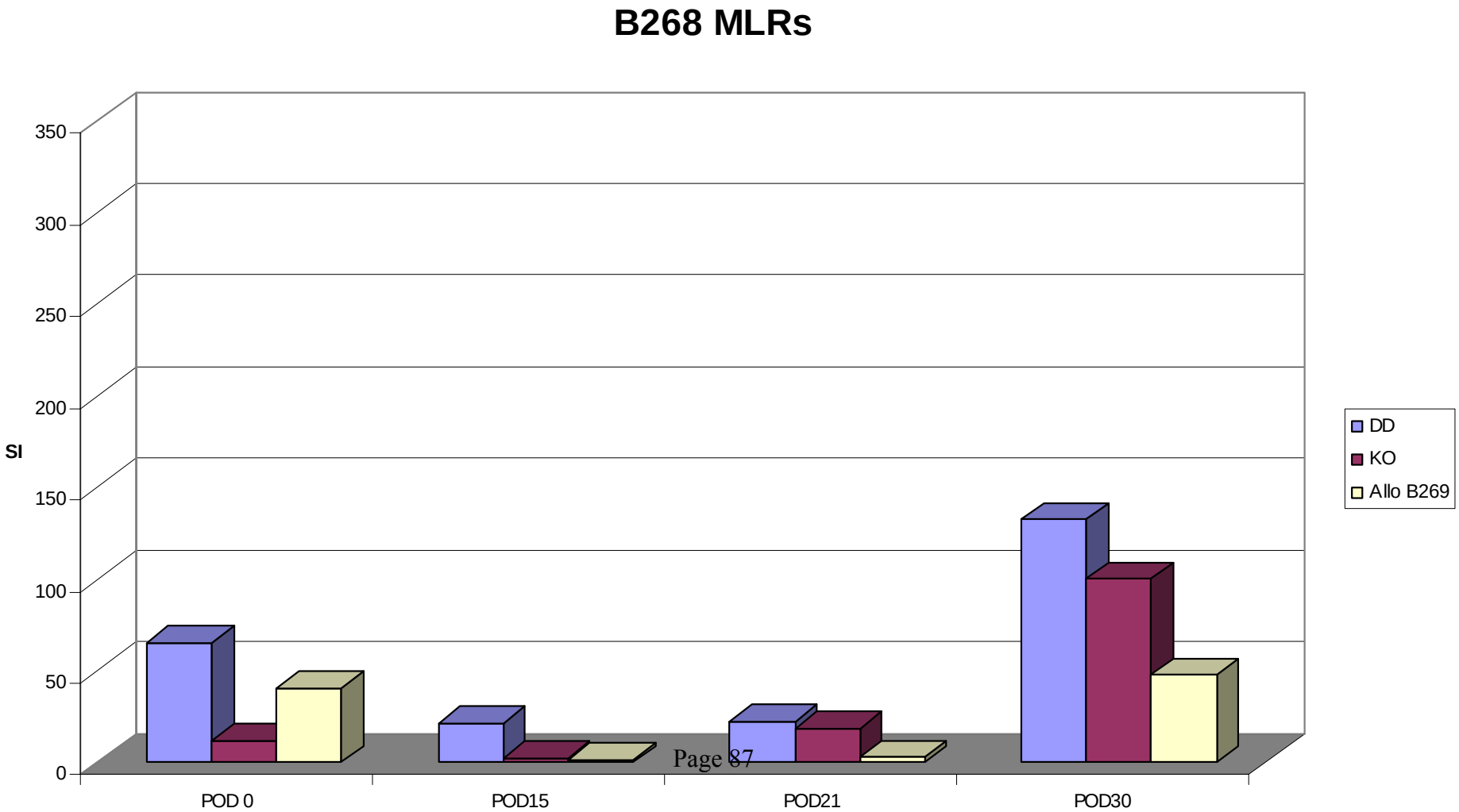
Day 30

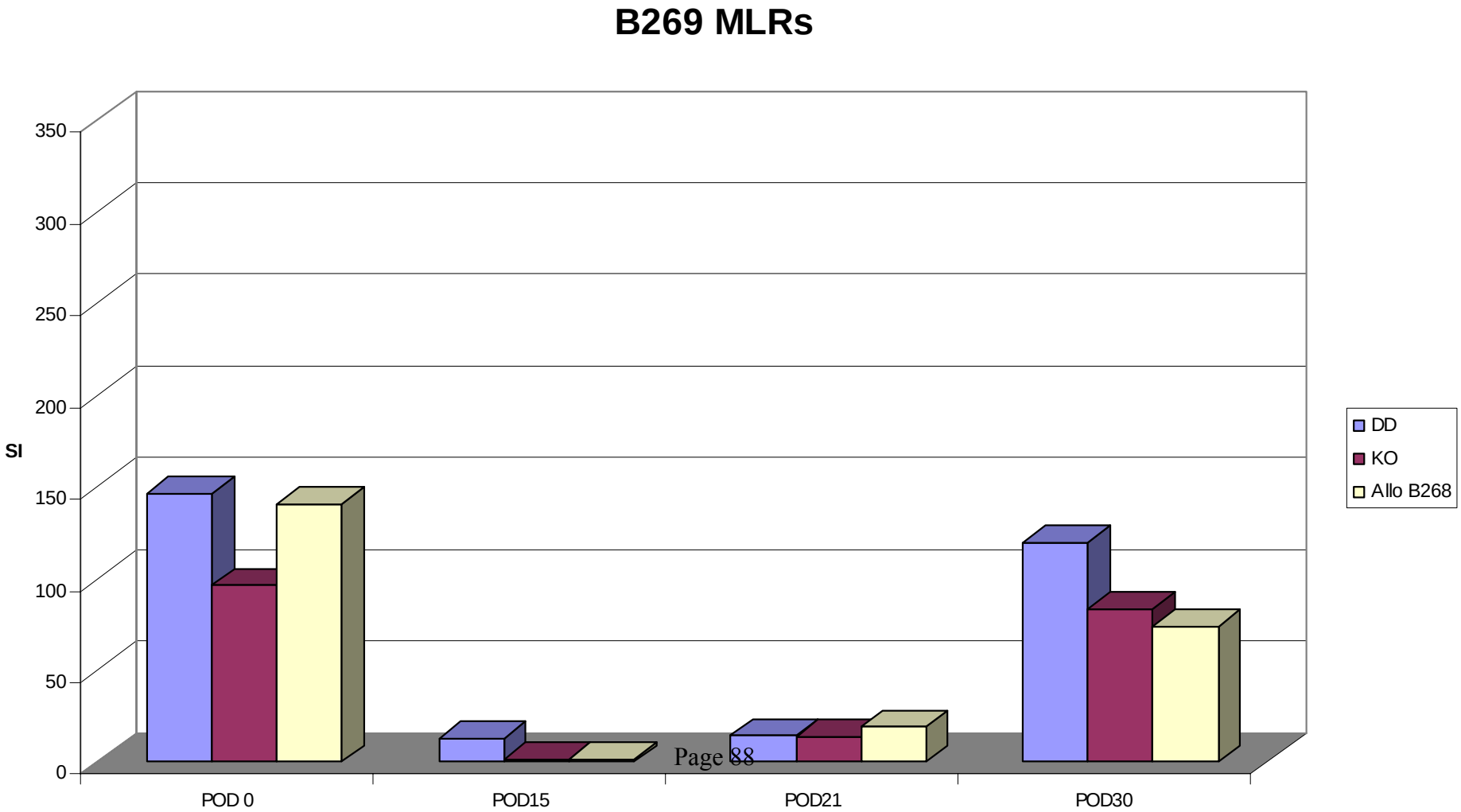
II set of skin grafts

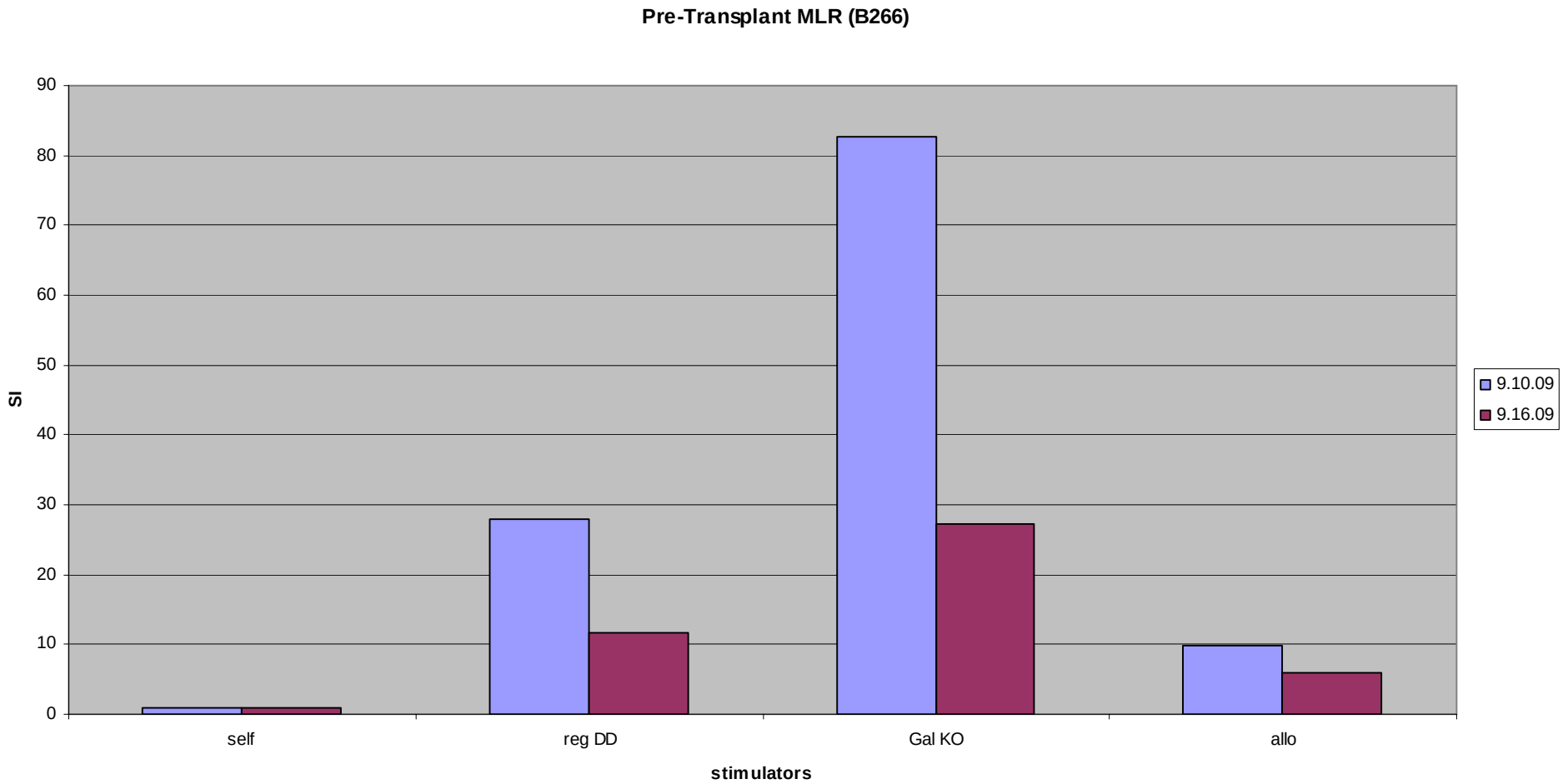



B266 MLRs
SI
350
300
250
200
150
100
50
0
POD 0
POD21
POD30
Pre-II set
POD7
POD14
POD21
POD30
DD
KO
Allo

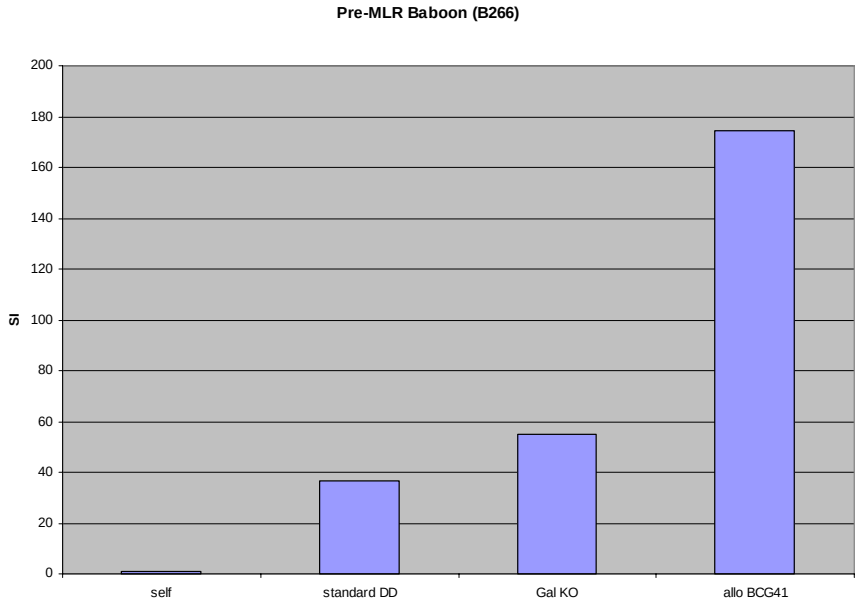

B267 MLRs
SI
350
300
250
200
150
100
50
0
POD 0
POD21
POD30
Pre-II set
POD7
POD14
POD21
POD30
DD
KO
Allo

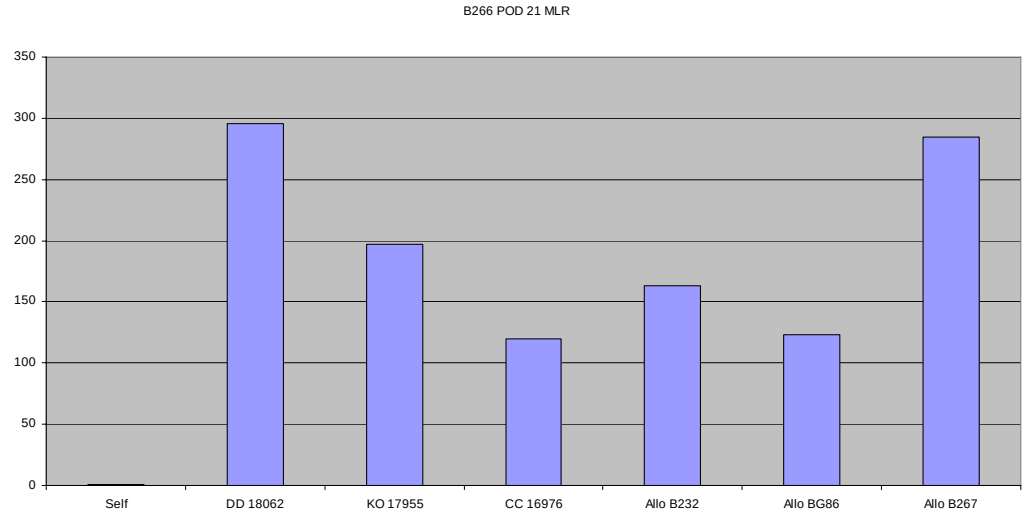

B268 MLRs
SI
350
300
250
200
150
100
50
0
POD 0
POD15
POD21
POD30
DD
KO
Allo B269

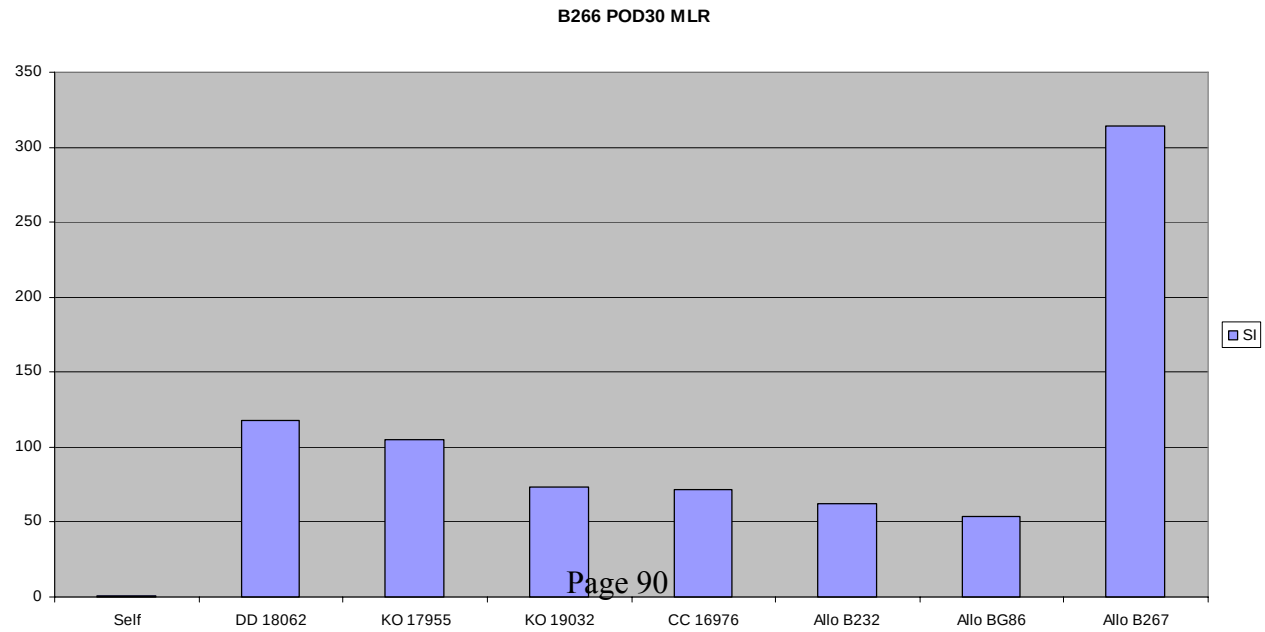

B269 MLRs
SI
350
300
250
200
150
100
50
0
POD 0
POD15
POD21
POD30
DD
KO
Allo B268


Pre-Transplant MLR (B266)
SI
90
80
70
60
50
40
30
20
10
0
self
reg DD
Gal KO
allo
stimulators
9.10.09
9.16.09

B266 MLRs (Self, Allo, GalT +, GalT-KO)

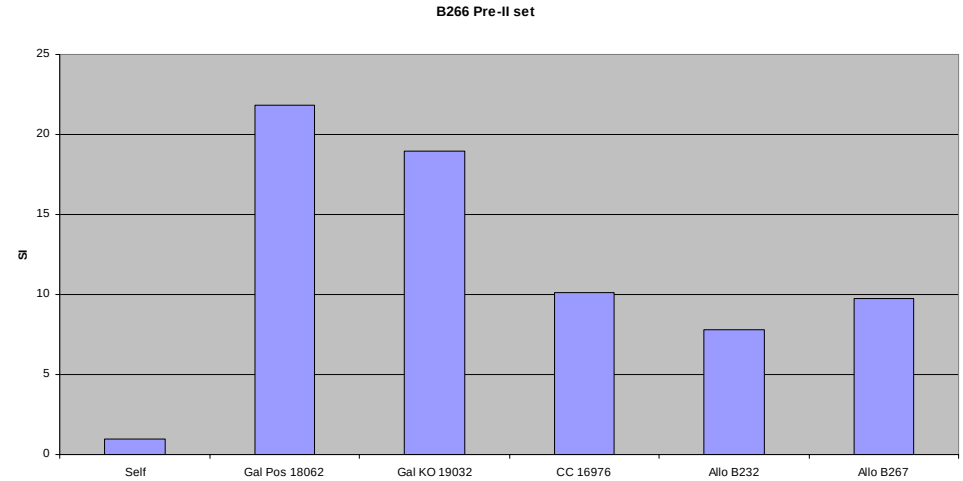


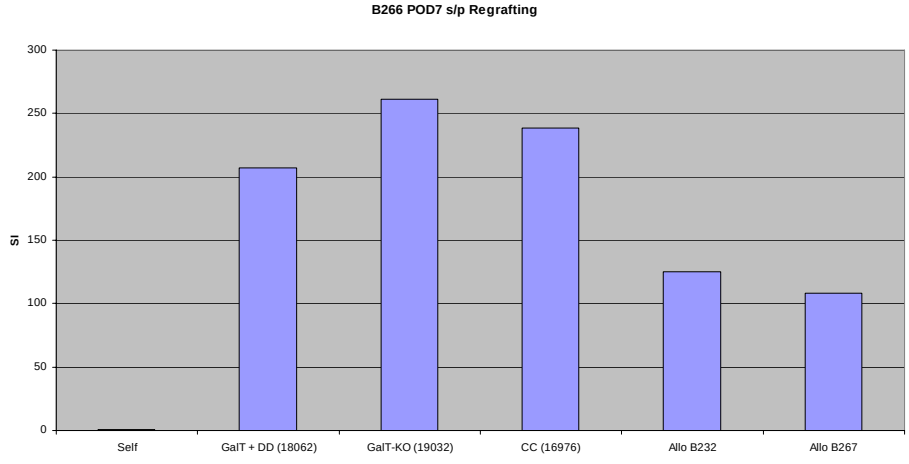


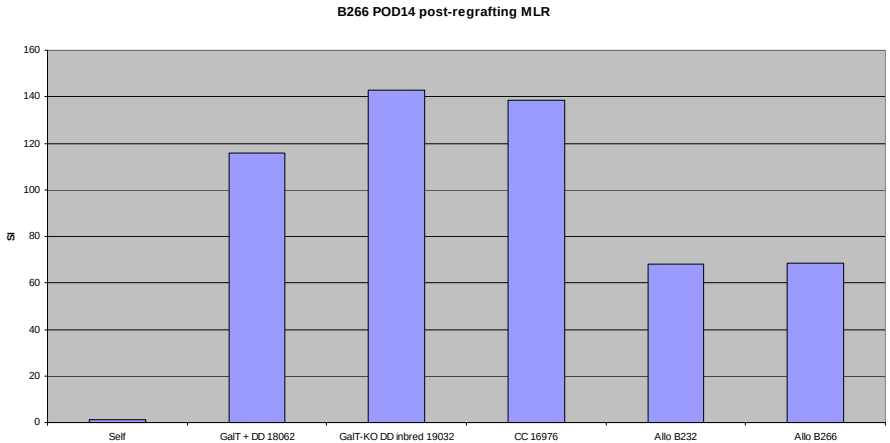


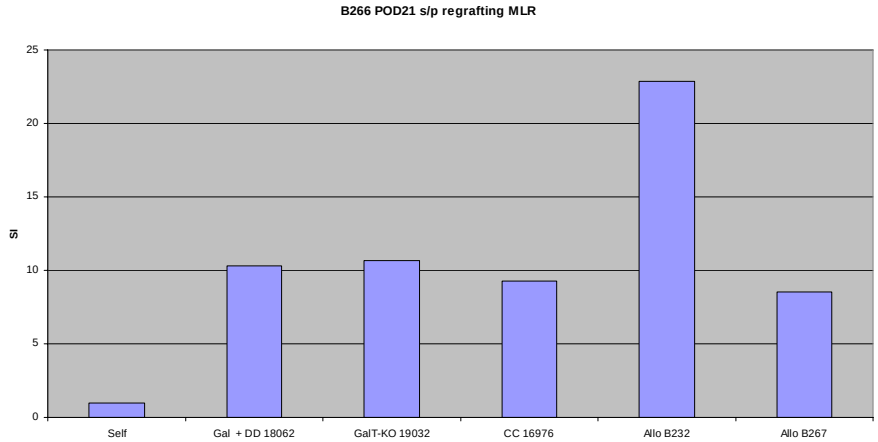
No CyA

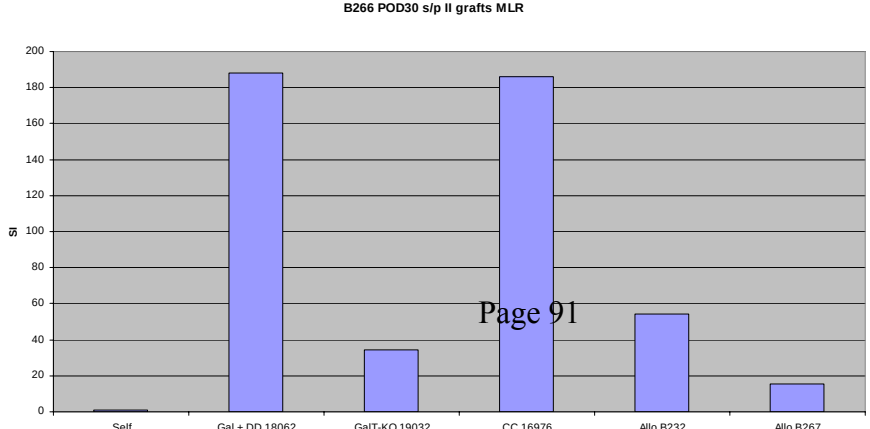
B266 MLRs (following regrafting with Self, Allo, GalT-KO fresh, Gal KO frozen)





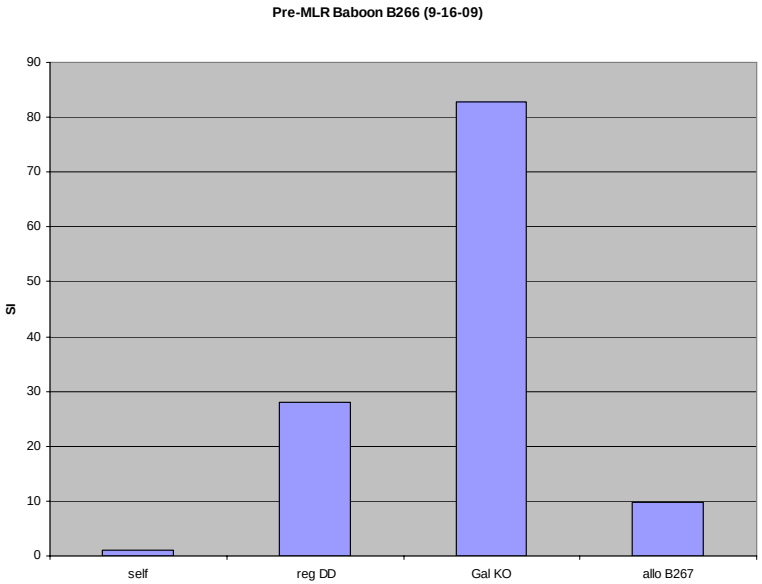


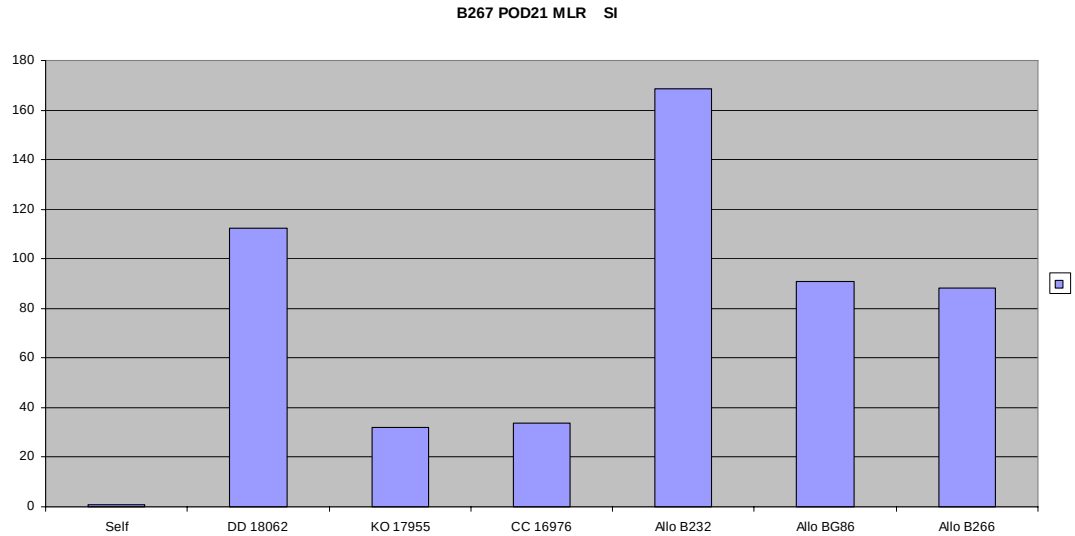


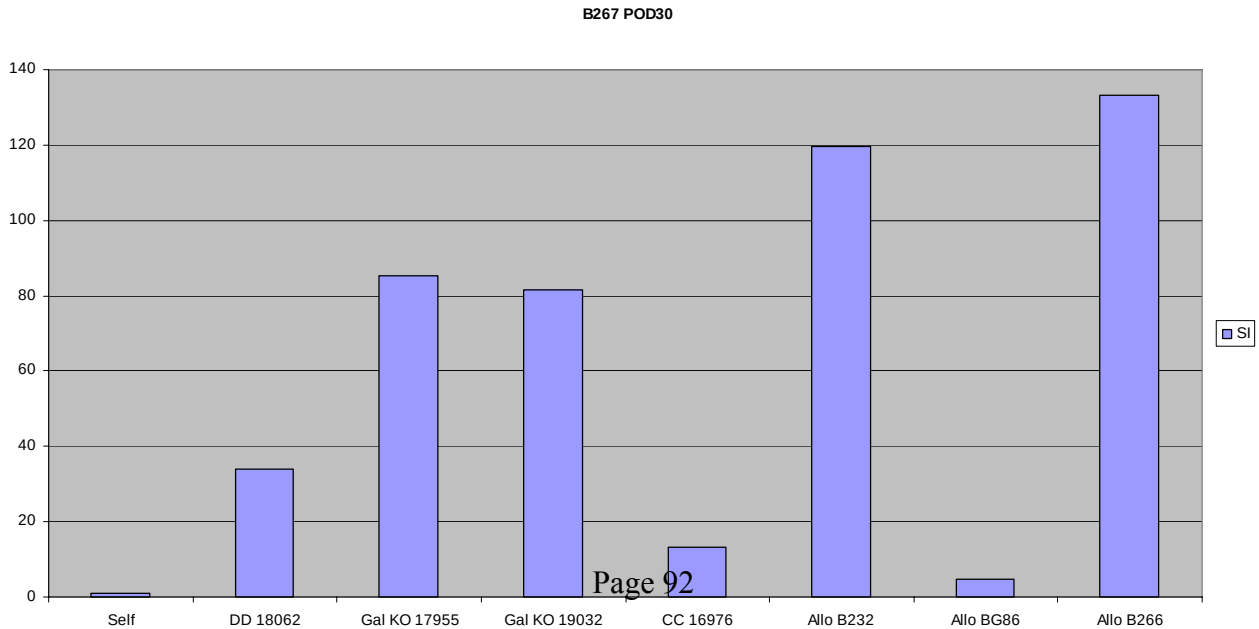


No CyA

B267 MLRs (Self, Allo, GalT +, GalT-KO)

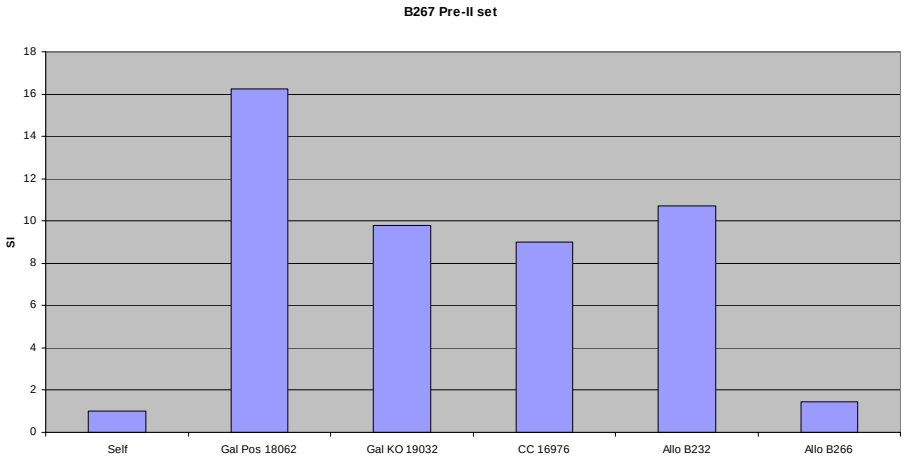


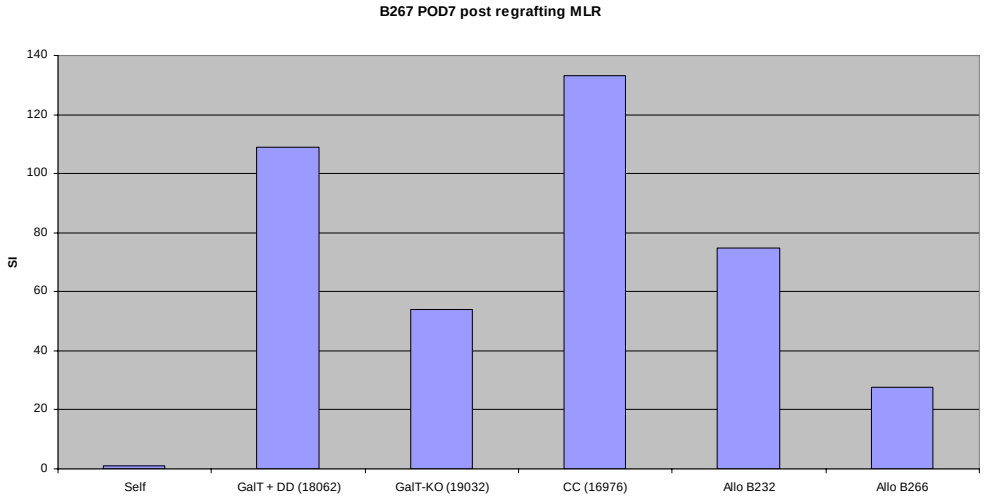


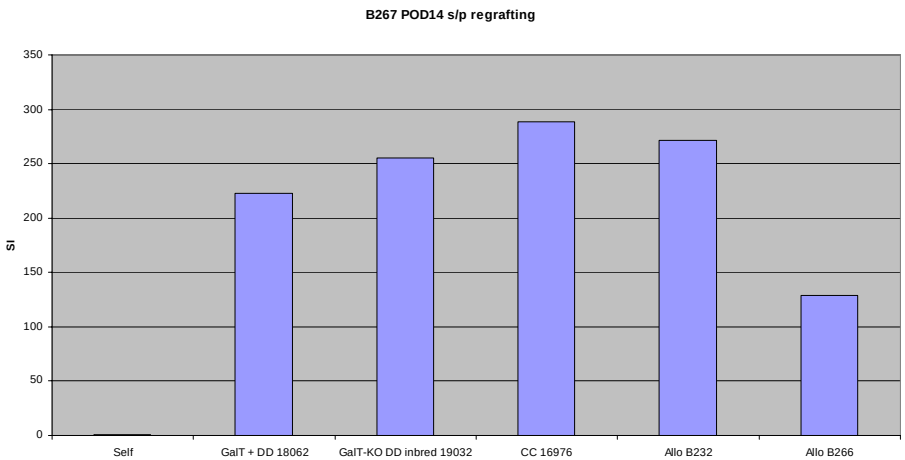


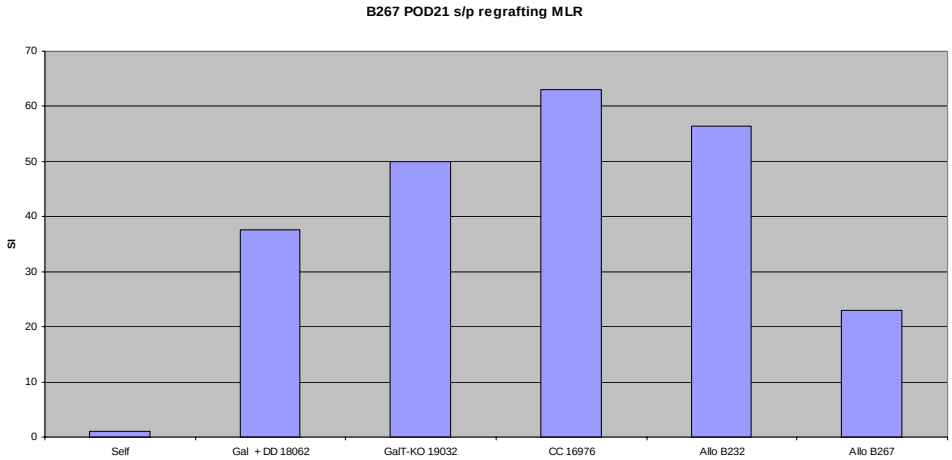
CyA

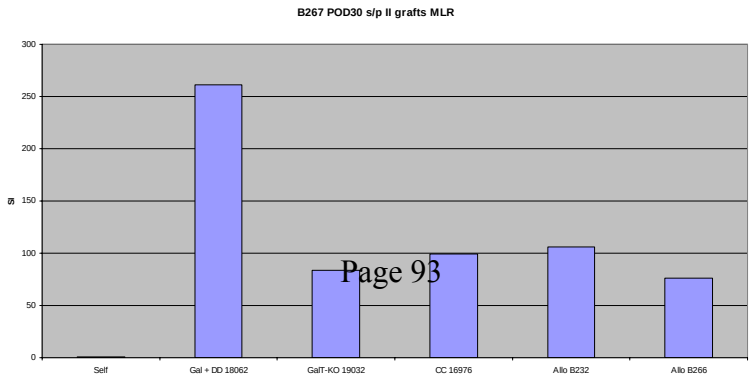
B267 MLRs (following regrafting with Self, Allo, GalT-KO fresh, Gal KO frozen)





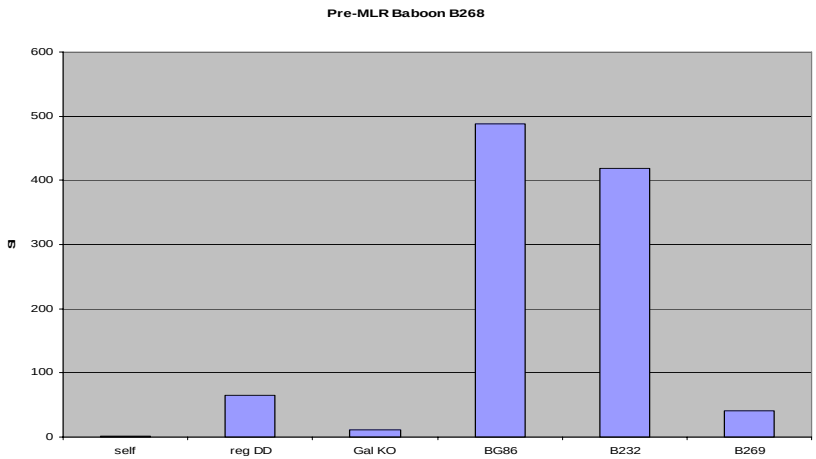


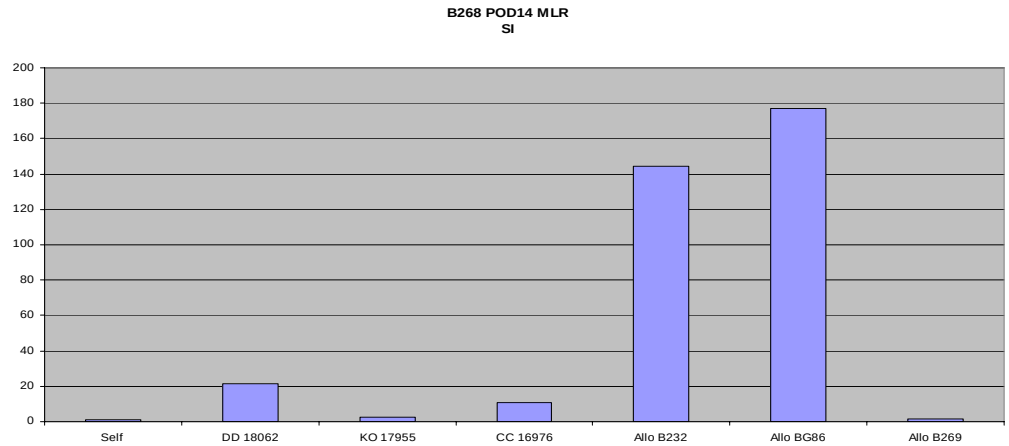


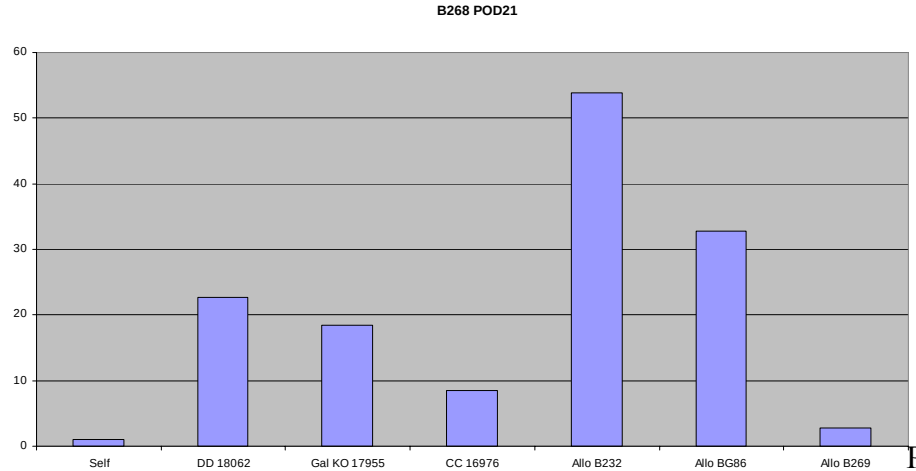


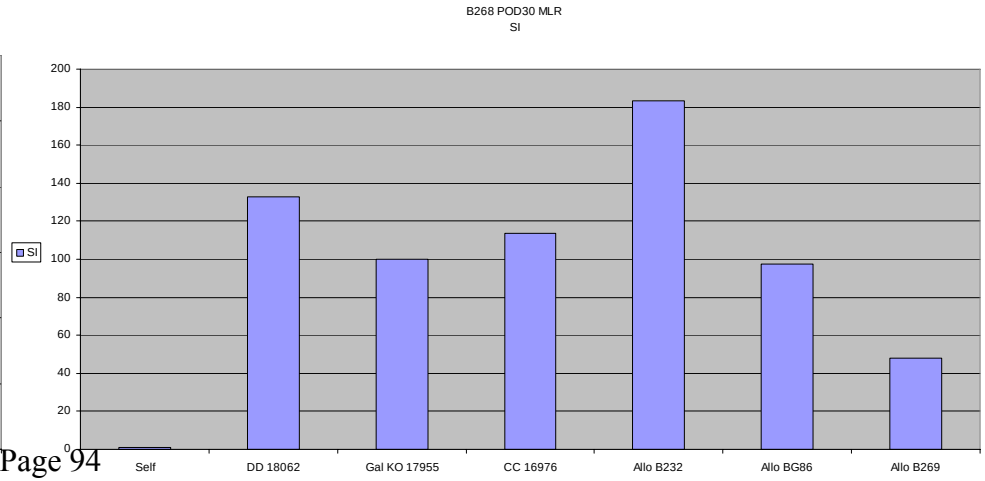
CyA

B268 MLRs (Self, Allo, GalT-KO)

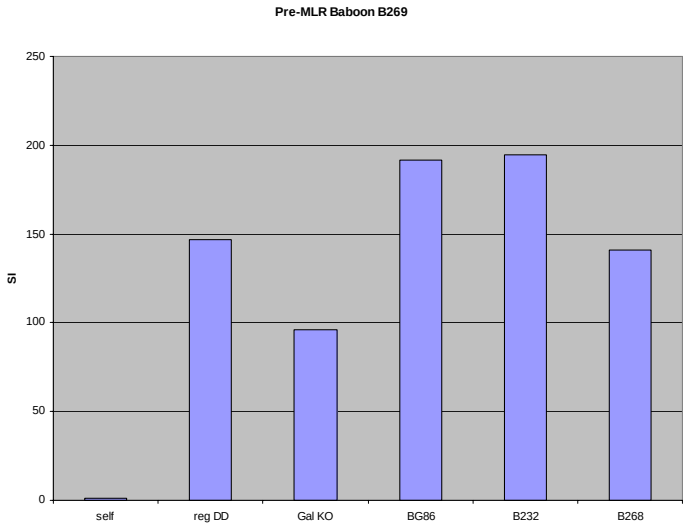


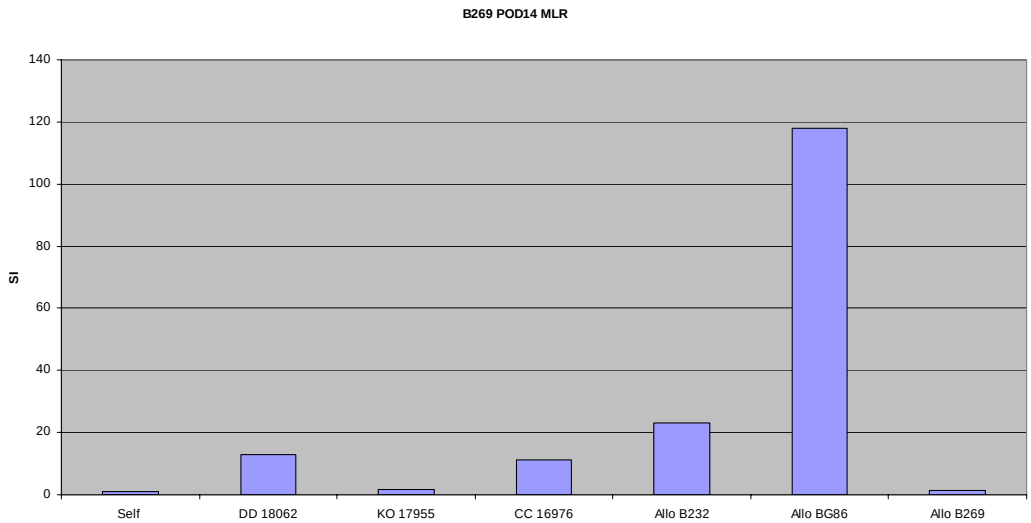


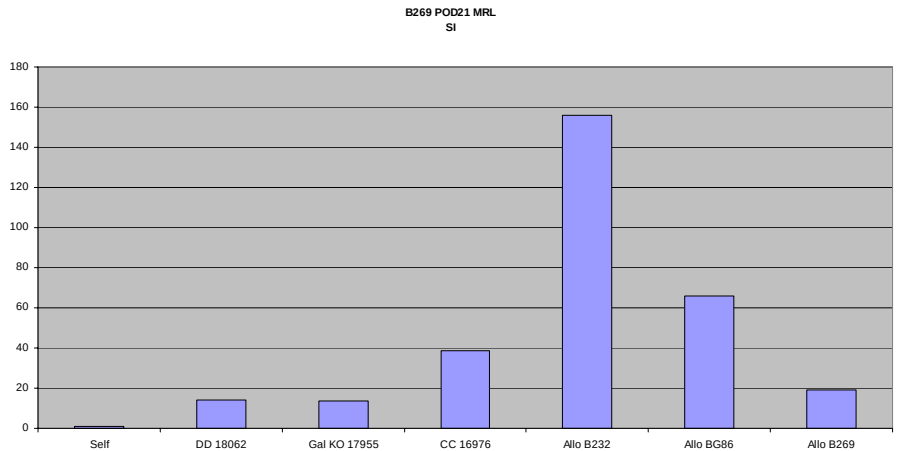


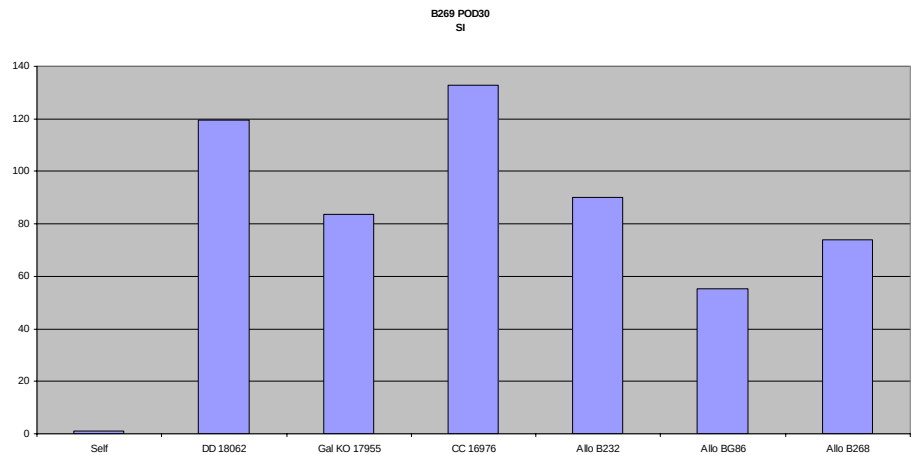


B269 MLRs (Self, Allo, Gal +)

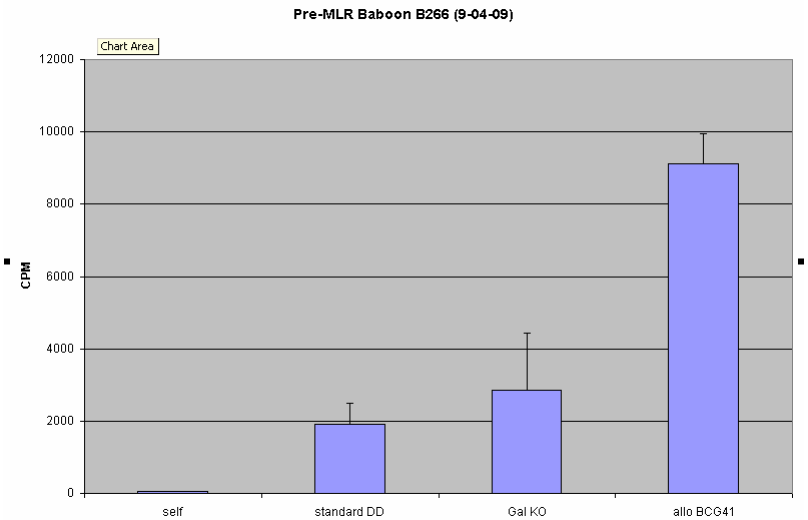


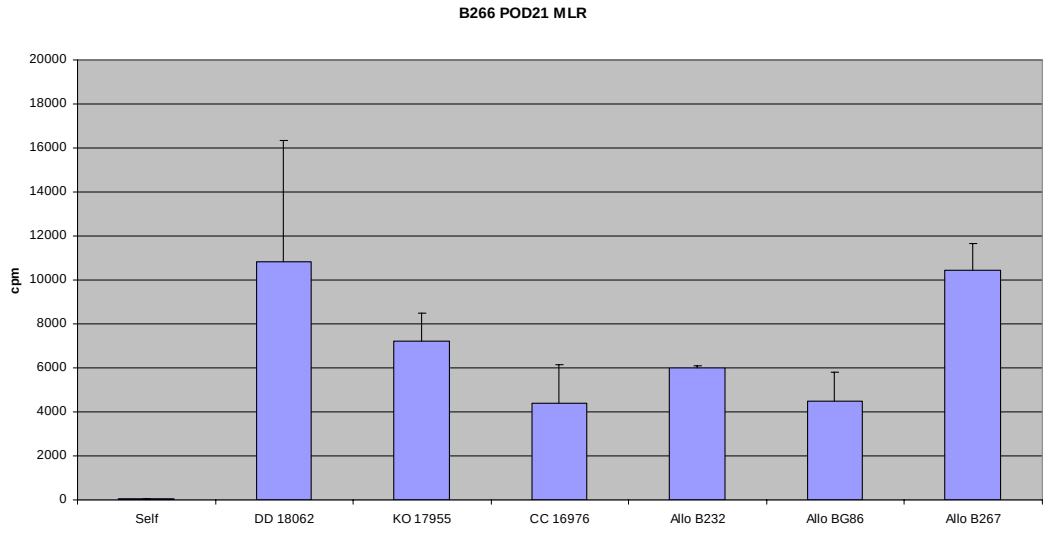


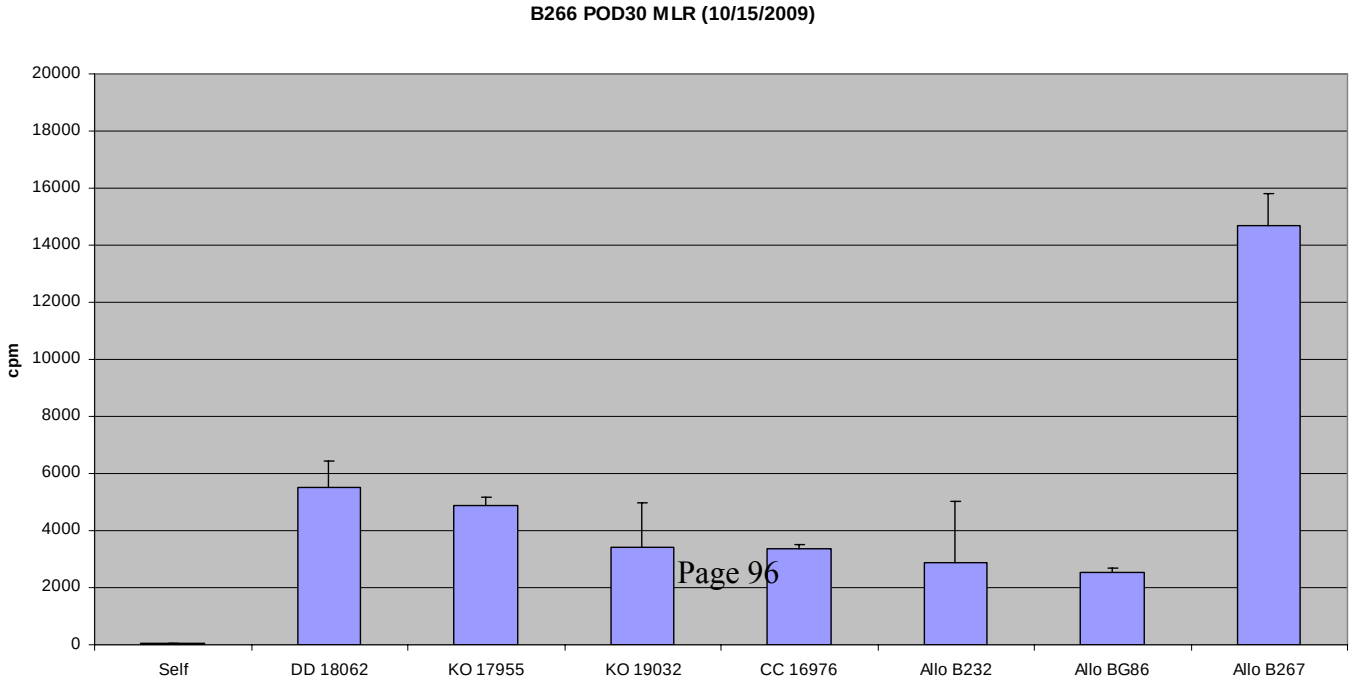


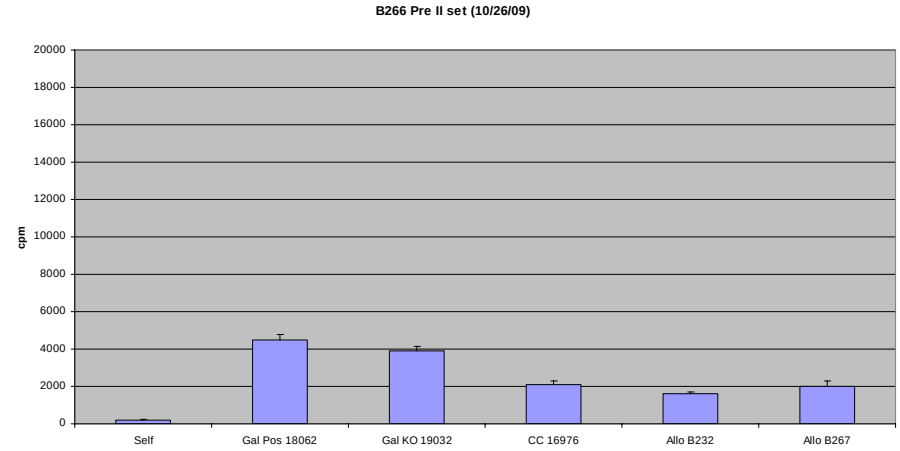


B266 MLRs (Self, Allo, GalT +. GalT-KO)





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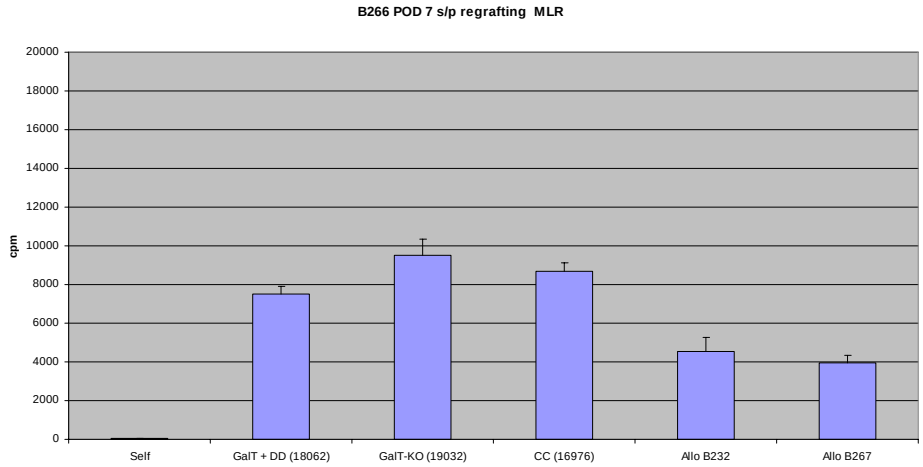
Gal KO 19032
CC 16976
Allo B232
Allo B267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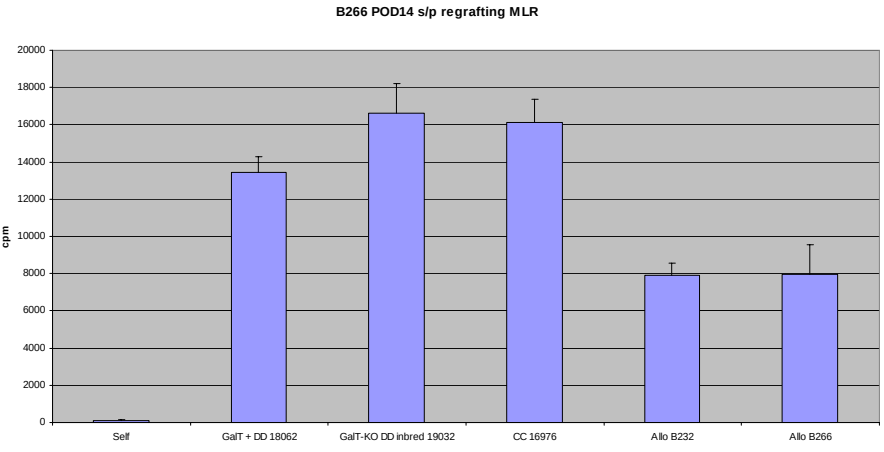
GalT + DD (18062)
GalT-KO (19032)
CC (16976)
Allo B232
Allo B267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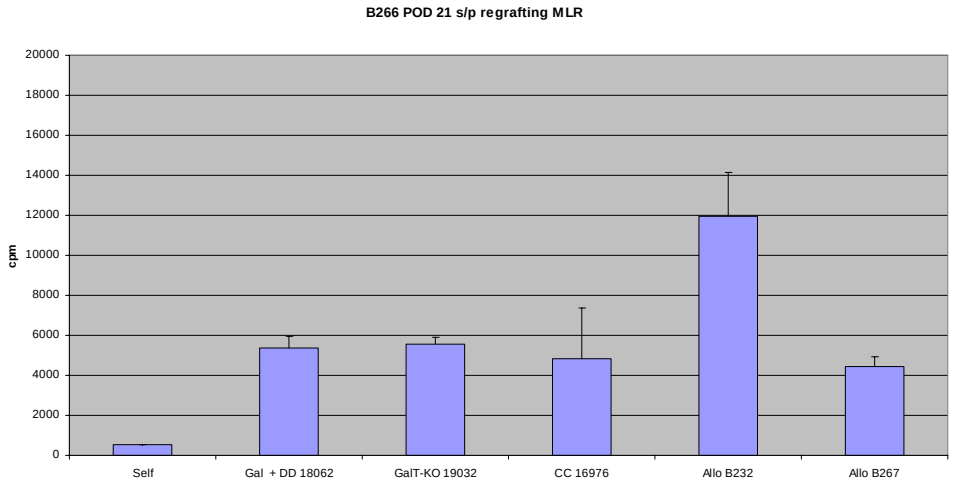
Allo B266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GalT-KO 19032
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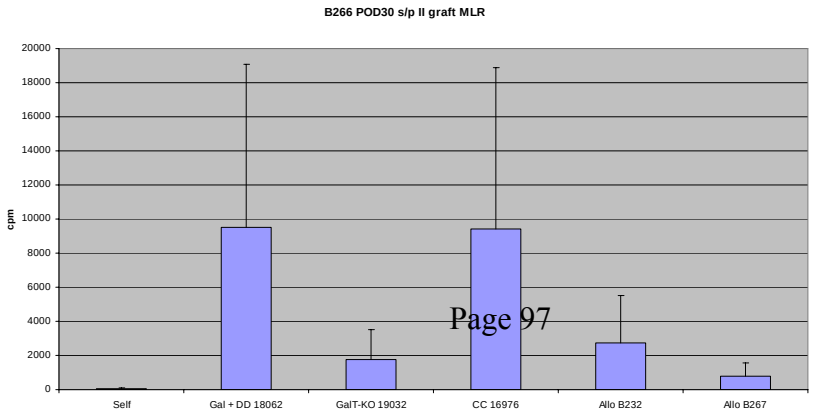
Allo B232
Allo B267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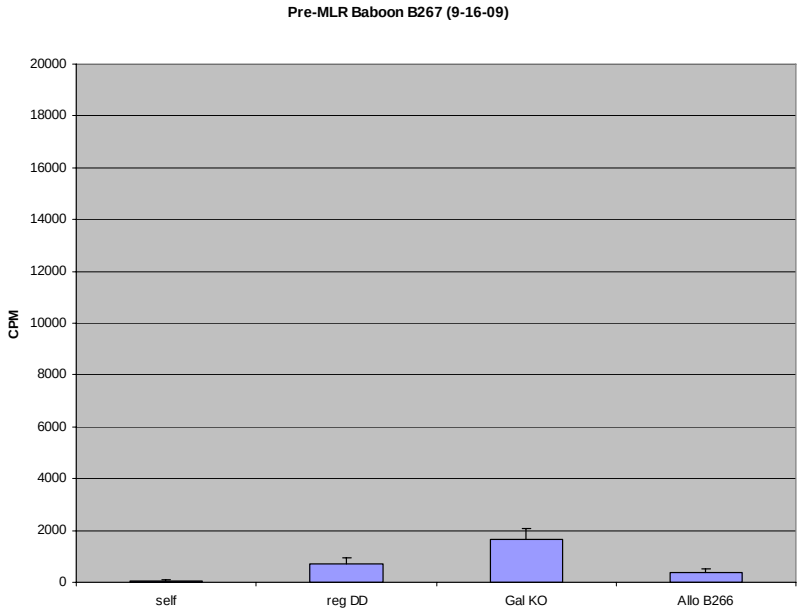
Gal + DD 18062
GalT-KO 19032
CC 16976
Allo B232
Allo B267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2000
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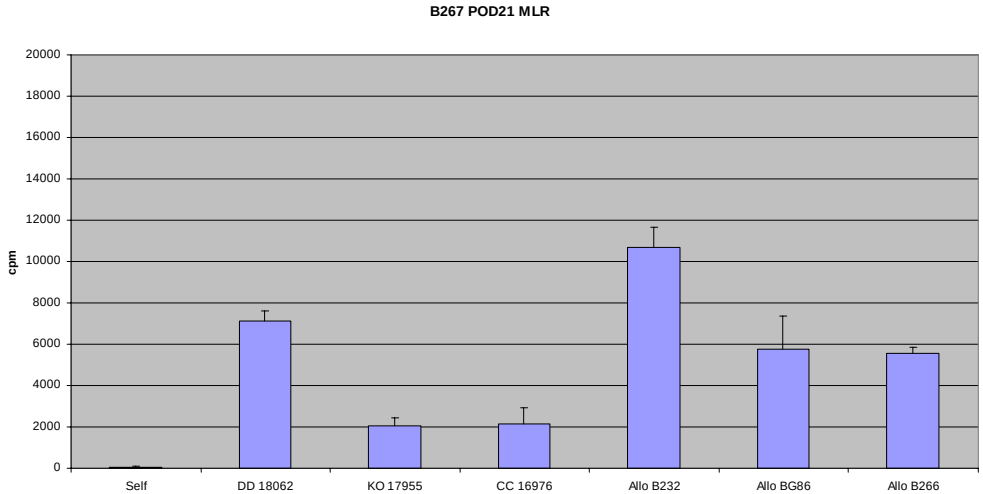
reg DD
Gal KO
Allo B266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KO 17955
CC 16976
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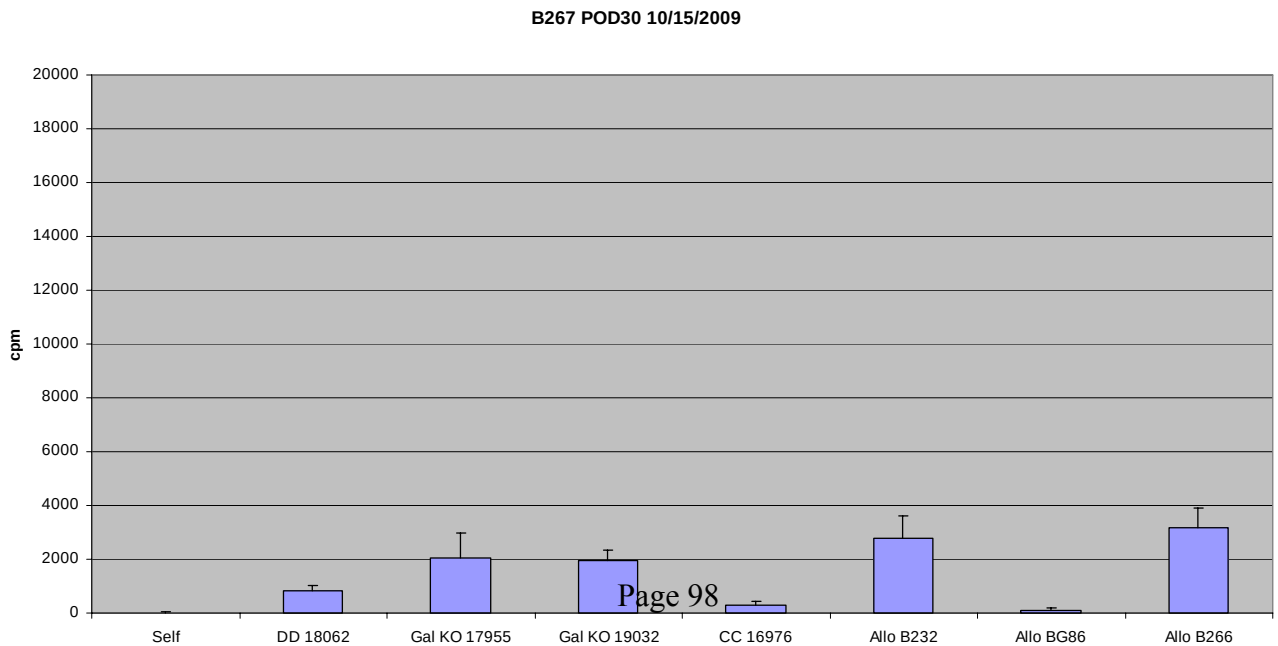
Allo BG86
Allo B266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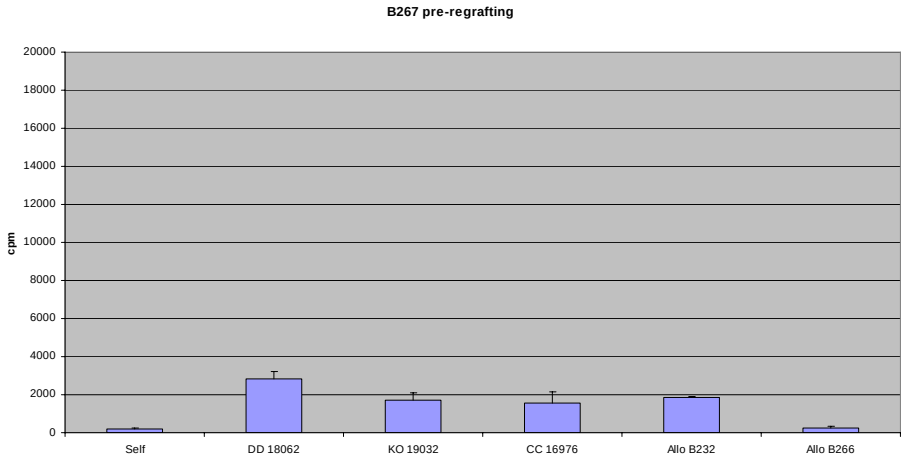
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KO 19032
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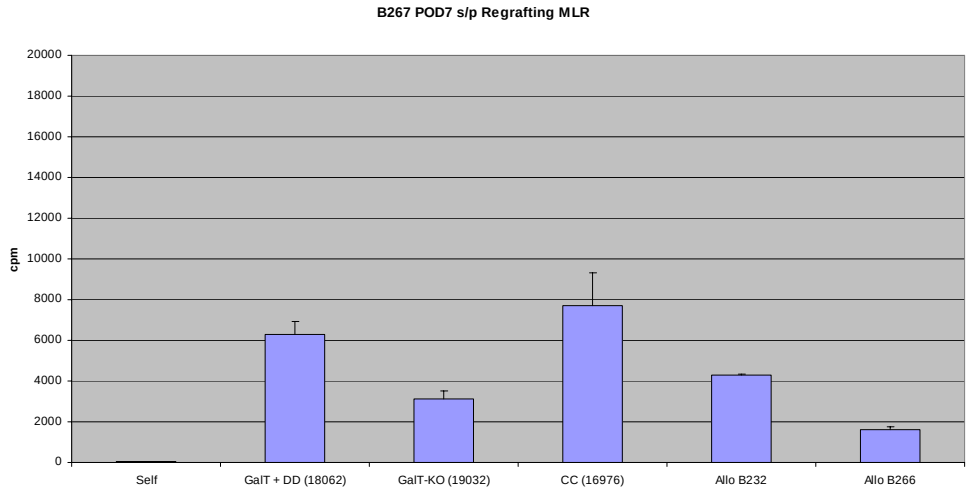
Allo B266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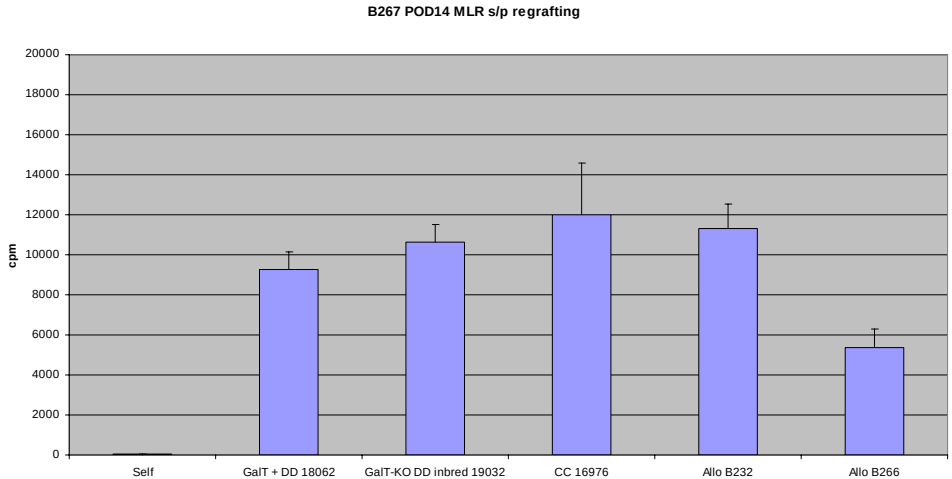
CC (16976)
Allo B232
Allo B266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GalT-KO DD inbred 19032
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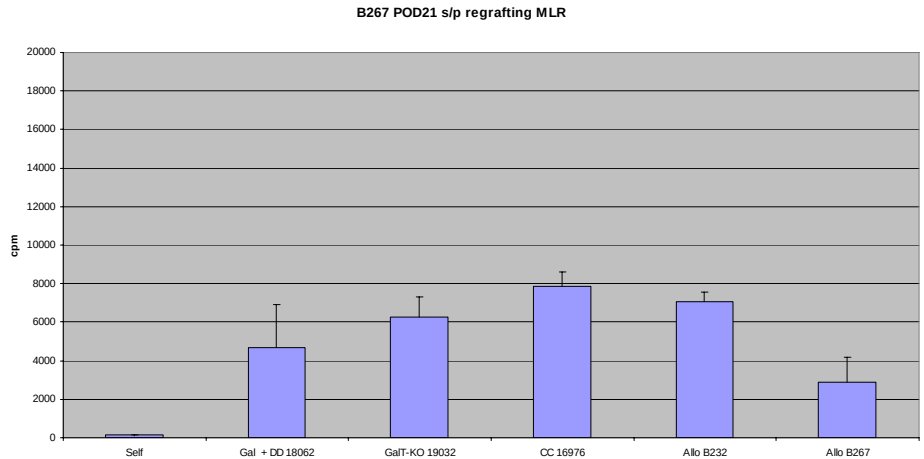
Allo B266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4000
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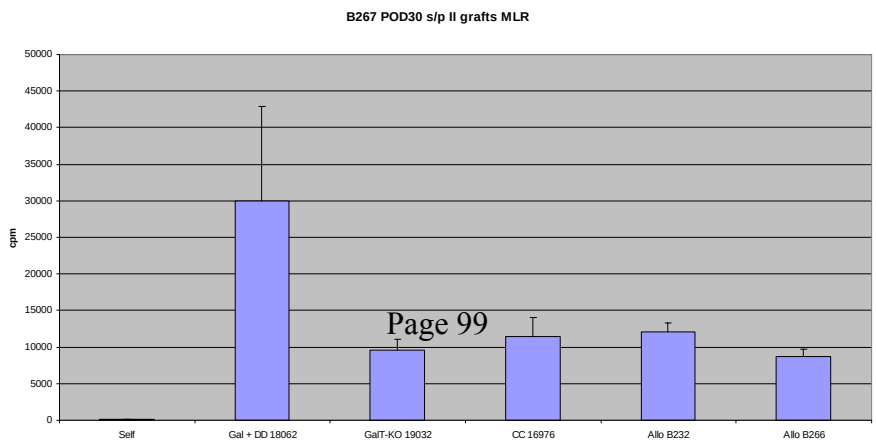
Gal + DD 18062
GalT-KO 19032
CC 16976
Allo B232
Allo B267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Gal + DD 18062
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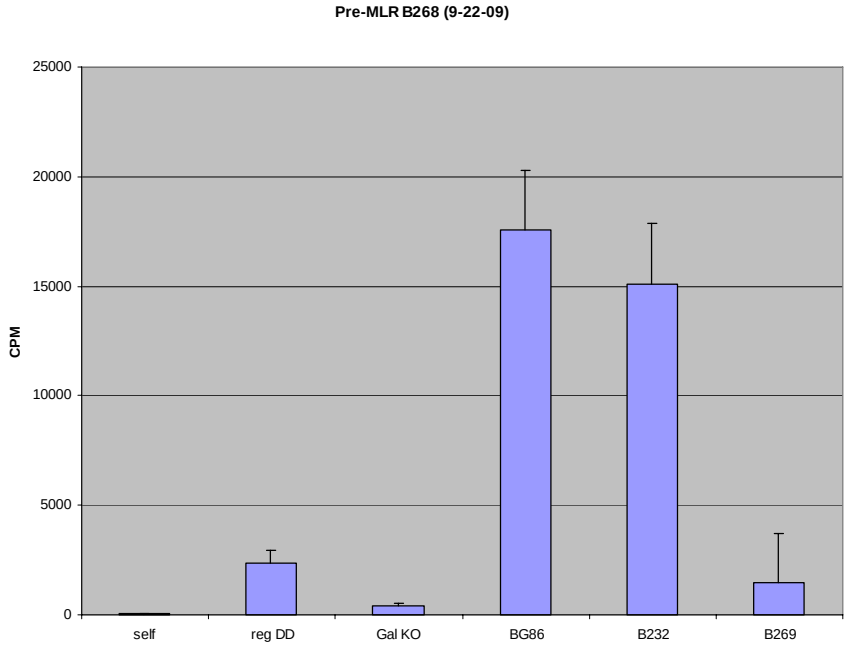
Allo B266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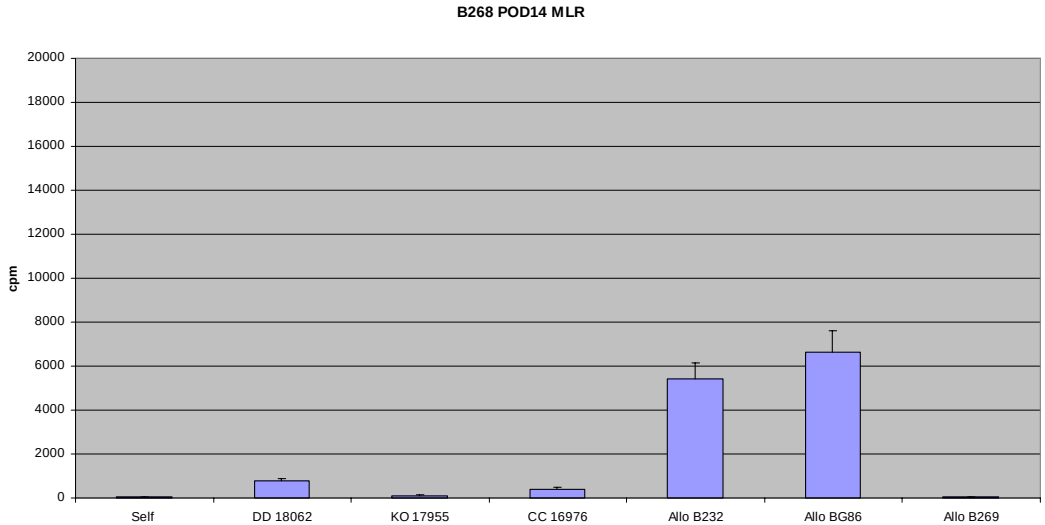
reg DD
Gal KO
BG86
B232
B269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CC 16976
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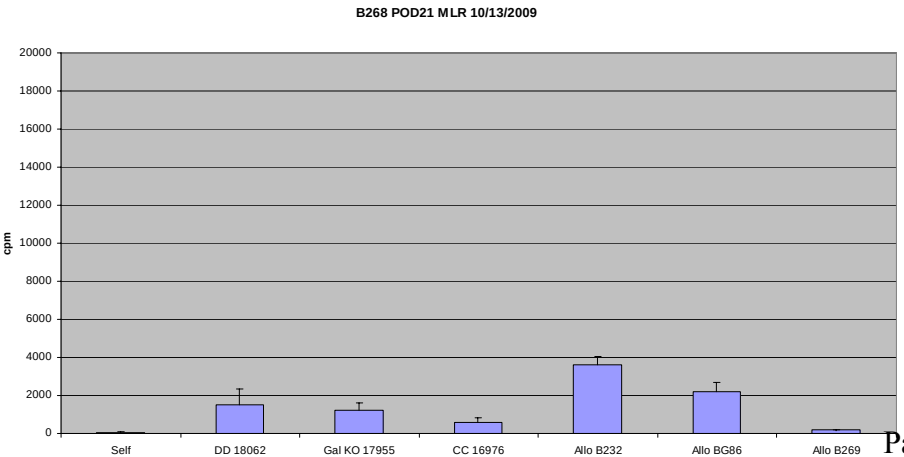
Allo BG86
Allo B269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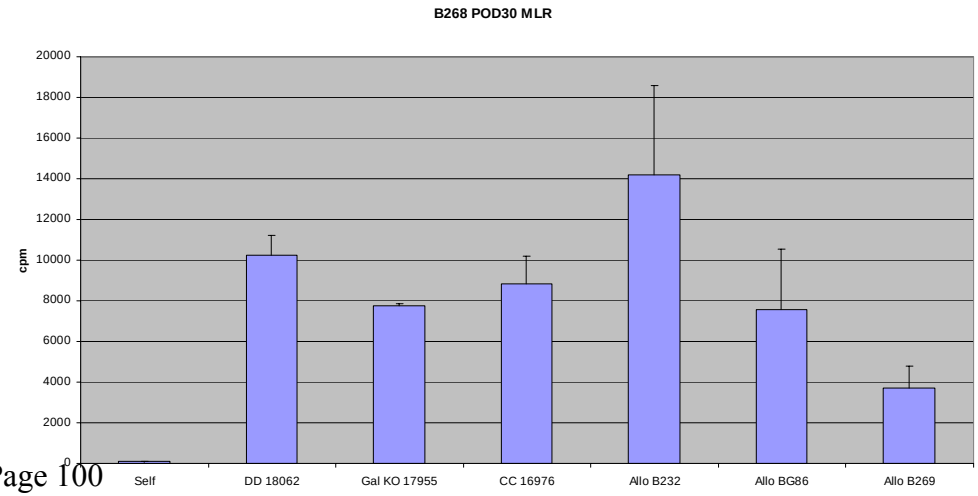
Allo B269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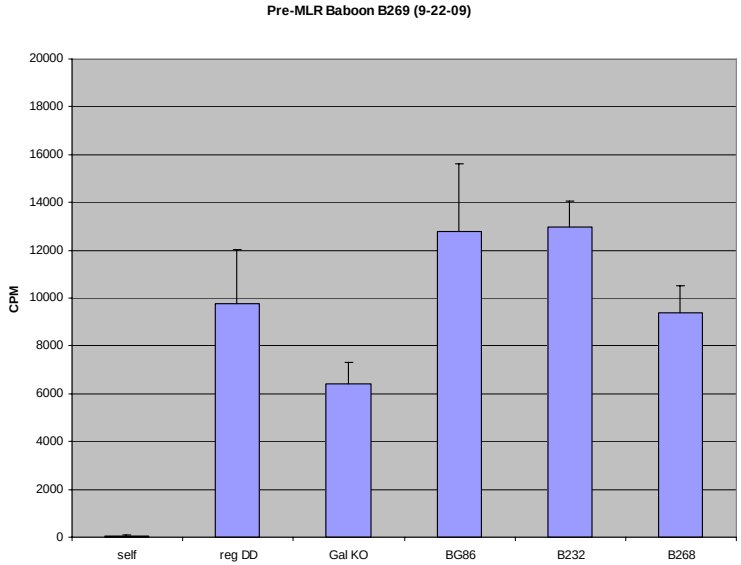
Allo BG86
Allo B269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B232
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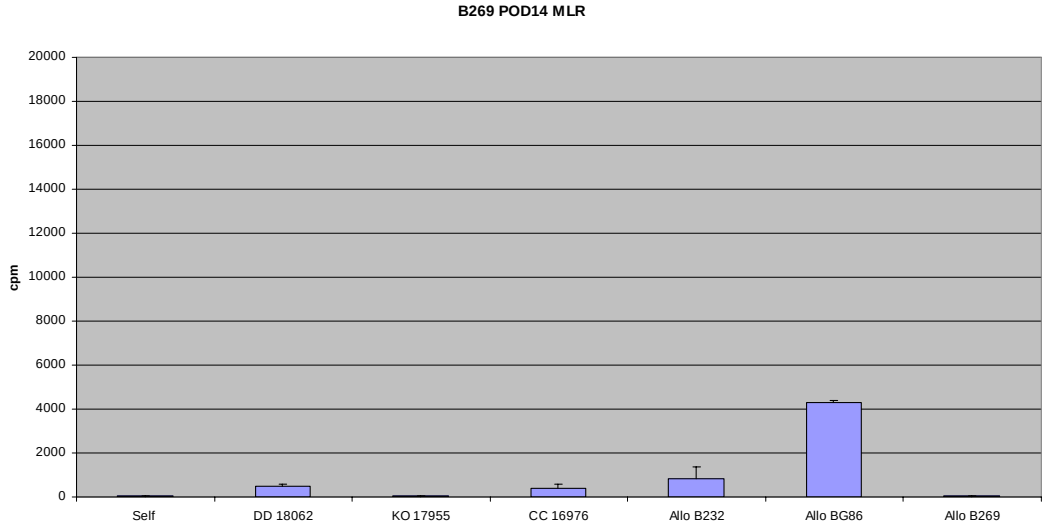
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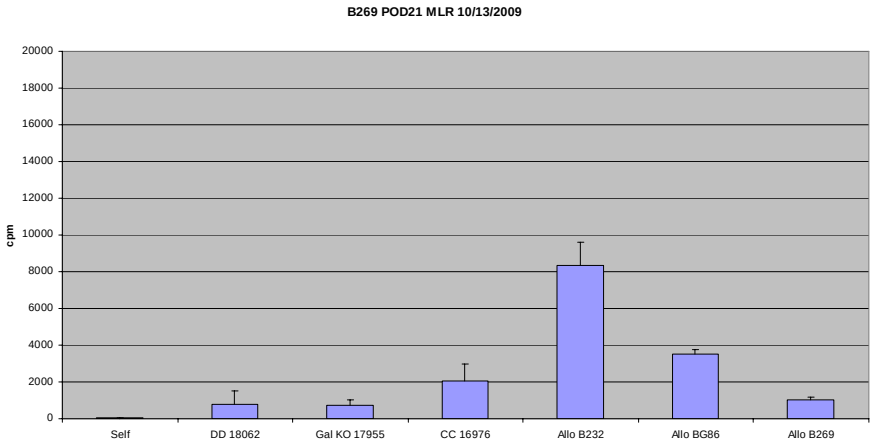
Allo BG86
Allo B269


B269 POD21 MLR 10/13/2009
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CC 16976
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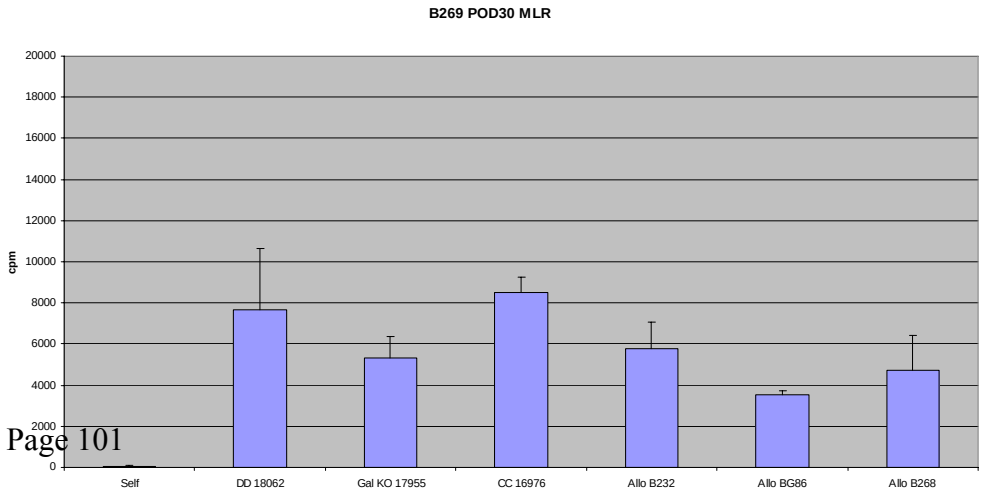
Allo BG86
Allo B269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Allo B232
Allo BG86
Allo B268

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Short Communication

Prolonged survival of GalT-KO swine skin on baboons

Weiner J, Yamada K, Ishikawa Y, Moran S, Etter J, Shimizu A, Smith RN, Sachs DH. Prolonged survival of GalT-KO swine skin on baboons. Xenotransplantation 2010; 17: 147–152. © 2010 John Wiley & Sons A/S.

Abstract: Background: Allogeneic skin is currently the best alternative to autologous skin as a temporary treatment for severe burns, but it has several drawbacks. As a potential alternative, we have evaluated GalT-KO swine skin, which lacks expression of the Gal epitope, to investigate the effect of eliminating this epitope on survival of pig-to-baboon skin grafts.
Methods: Two adult baboons that had fully recovered from previous T cell depletion received simultaneous skin grafts from: (i) GalT-KO swine, (ii) Gal-positive swine, (iii) a third-party baboon, and (iv) self (control skin). Recipients were treated with cyclosporin for 12 days and the survival, gross appearance, and histology of the grafts were compared.
Results: In both baboons, the GalT-KO skin survived longer than either the Gal-positive swine skin or the allogeneic skin. Early rejection of the Gal-positive skin appeared to be mediated by cytotoxic preformed anti-Gal IgM antibodies, while the rejection of GalT-KO skin appeared to result from cellular mechanisms.
Conclusions: GalT-KO skin may have potential clinical benefits as an alternative to allogeneic skin as a temporary treatment for severe skin injuries.

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Key words: allografts – galT-KO – skin – xenografts – xenotransplantation

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Introduction

According to the American Burn Association, there are approximately 500 000 burn injuries per year in the United States, with roughly 40 000 requiring hospitalization [1].

A treatment option that has helped to decrease mortality over the past 10 yrs has been the immediate excision of burned skin with replacement by grafted skin [2–4]. The ideal material for grafting is autologous skin, taken from a non-burned region of the patient's own skin. The supply of healthy autologous skin, however, is limited in severely burned patients, even when expansion techniques, such as ''meshing,'' are used [5,6]. Allogeneic skin is considered the gold standard for temporary grafts [1]. In addition, it is able to engraft temporarily before rejection occurs, and it can be frozen and stored for transportation or later use. However, disadvantages include ethical concerns, cost considerations, and possibility of disease transmission, and like all types of temporary grafts, it is more easily infected than autologous skin and not always available.

Pig skin is known to have many characteristics similar to that of humans [7–12] and glutaraldehyde-fixed pig skin has been utilized as a temporary cover for third degree burns under battlefield conditions [13]. The properties of such fixed skin are far inferior to those of living skin, and living pig skin is susceptible to rapid rejection, thought to be due, at least in part, to natural antibodies present in all humans [14,15]. The recent development in this laboratory of genetically modified swine missing the Gal epitope, the major cell surface determinant toward which these antibodies are directed, made it possible that skin from these ''GalT-KO'' animals might provide a new source of living skin grafts for the immediate treatment of burns. Previous studies in our laboratory have shown that the use of GalT-KO swine donor organs has greatly increased the survival of vascularized xenograft organs in baboon recipients [16,17].

In an attempt to evaluate whether the use of skin from GalT-KO swine would be of benefit in prolonging the survival of pig-to-primate skin grafts, we transplanted GalT-KO skin onto two baboon recipients and compared the survival of these grafts with that of Gal-positive and allogeneic grafts. We report here the results of this preliminary study.

Materials and methods

Animals

Two 3- to 4-yr-old baboons that were available from a previous study were used as recipients for this initial experiment. Both animals had been thymectomized and treated with an anti-T cell immunotoxin in the previous protocol and then followed for several months, during which time all immunologic parameters returned to baseline, including natural antibodies as well as numbers and phenotypes of white blood cells in both the peripheral blood and lymph nodes.

Allogeneic skin donors were unrelated baboons available in our animal facility. Xenogeneic donors were from our closed herd of MGH Miniature Swine. Animals from the standard line of SLA^{dd}, $GalT^{+/+}$ miniature swine [18] or from our $GalT^{-/-}$ (GalT-KO) line, derived from this standard inbred line [19], were used.

Surgery

Harvesting of donor skin was performed using a Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ, USA), with depth set at 24 mm. Anesthesia consisted of induction with 2 mg/kg ketamine i.m. followed by maintenance with isoflurane administered by mask. Partial thickness sections of skin (approximately 3×5 inches) were taken. Grafts were stitched into place with interrupted 1-0 sutures and covered with a Duoderm dressing for 2 days, after which they were left open, protected by a loose fitting jacket. Recipients were treated with 13 mg/kg cyclosporine intramuscularly for 12 days.

Biopsies

Recipients were sedated and anesthetized to evaluate the skin grafts and draw blood at various times postoperatively. On each of these occasions, grafts were examined, graded, cleaned, and photographed, and blood was drawn for complete blood count, serum collection, and in vitro assays. At selected times, 6.0-mm full-thickness punch biopsies were taken for histologic evaluation of frozen and formalin samples.

PBMC isolation

For separation of peripheral blood leukocytes, freshly heparinized whole blood was diluted 1 : 2 with Hank's balanced salt solution (HBSS; GIBCO BRL, Gaithersburg, MD, USA) and the mononuclear cells were obtained by gradient centrifugation using lymphocyte separation medium (Organon Teknika, Durham, NC, USA) as previously described [20] and stored in mixed leukocyte reaction (MLR) media.

Histology

Biopsy specimens were either fixed in 10% buffered formalin or immediately frozen in liquid nitrogen. Fixed samples were embedded in paraffin, and 4-μ sections were stained with hematoxylin and eosin. Immunohistochemical analysis of frozen samples was carried out using the avidin–biotin horseradish–peroxidase complex technique [21].

Complement-mediated cytotoxicity

Cytotoxic antibodies to Gal-positive and GalT-KO PBMC were detected by complement-mediated cytotoxic assays, as previously described [22]. Briefly, target cell suspensions were diluted to 5×10^6 cells/ml in Medium 199 (Cellgro, Herndon, VA, USA) supplemented with 2% fetal calf serum and serially diluted from 1 : 2 to 1 : 1024. In some cases, IgM was eliminated prior to the assay by adding dithiothreitol (DTT; Sigma–Aldrich, St. Louis, MO, USA) to the serum. In 96-well U-bottom plates (Costar, Cambridge, MA, USA), 25 μl of the appropriate target cell suspension was incubated with 25 μl of diluted serum or controls for 15 min at 37 °C, followed by a second incubation with 25 μl of appropriately diluted rabbit complement. Dead cells were identified by staining for 30 min with 10 μl of 7-AAD. Data were acquired, and the percentage of dead cells was assessed, using a Becton–Dickinson FACScan (San Jose, CA, USA) and analyzed with WinList analysis software (Verity Software House, Topsham, ME, USA).

Results

Gross findings following skin grafts

On baboon 1, the four skin grafts (self, GalT-KO, Gal positive, and allo baboon, left to right, respectively in Fig. 1A) were placed side-by-side

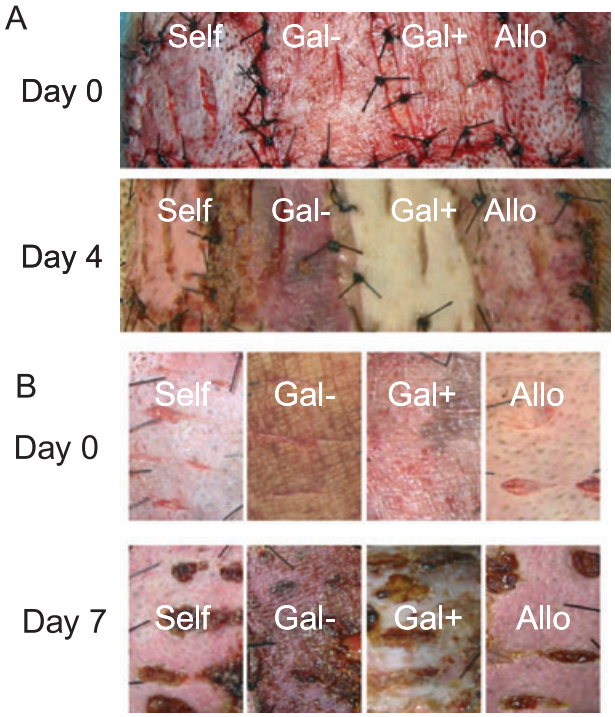

Fig. 1. (A) Baboon 1 skin grafts on days 0 and 4 (from left to right): self, GalT-KO, Gal-positive, allogeneic baboon. (B) Baboon 2 skin grafts on days 0 and 7 (from left to right): self, GalT-KO, Gal-positive, and allogeneic baboon.

on a single graft bed and covered with bacitracin and a gauze dressing. On baboon 2 (Fig. 1B), the graft beds were prepared separately to avoid spreading of local infection or inflammation from one graft to another, and no dressings were applied.

Baboon 1

By day 4, the self and allo grafts on baboon 1 were warm, soft, and pink, suggesting that they had engrafted and begun to re-vascularize. The GalT-KO skin was also warm and pink, although with slight mottling. In contrast, the Gal-positive pig skin was cool and white, suggesting a "white graft," as previously described for skin grafts that do not re-vascularize because of hyperacute rejection due to preformed antibodies [23,24]. By day 7, the self skin was still pink and healthy, but the allo skin had begun to develop a crust over the graft, as had the Gal-positive pig skin. The Gal-positive skin also appeared to be infected, producing purulent discharge, and the animal's white blood cell count rose. In contrast, the GalT-KO skin was warm, dark pink, and mostly intact, although localized, superficial infection caused some loss of integrity. The infection in the GalT-KO skin, which damaged part of the graft as well as a portion of the self graft, appeared to have spread from the neighboring infected Gal-positive skin. The animal was treated for 3 days with 15 mg/kg per day i.v. vancomycin, after which the superficial infection cleared and the white count returned to normal. By day 9, the allo skin had been totally rejected and was covered by scab, while a portion of the GalT-KO skin still remained intact. The self skin remained normal. The GalT-KO appeared to be totally rejected by day 11.

Baboon 2

On day 4, the self and allo grafts on baboon 2 were likewise warm, soft, and pink, whereas the Gal-positive graft was bright white and cool to touch. The GalT-KO skin was warm and soft, but with some purple mottling. There was minimal bleeding when the Gal-positive biopsy was taken, suggesting poor vascularization. The grafts appeared much the same on day 7 (Fig. 1B) except that a portion of the Gal-positive graft appeared grossly necrotic and purulent. By day 11, self skin was still warm, soft, and pink, but the allo skin was fully crusted with only half of it remaining intact. The Gal-positive skin was mostly crust with two small areas that remained bright white. The GalT-KO skin showed some moderate crusting at the edges, but otherwise remained soft and warm. By day 14, a small portion still appeared viable, and the final biopsy was taken from this portion.

Histologic findings following skin grafts

As seen in Fig. 2, the histology of the self skin graft on baboon 1 remained normal except for a small amount of non-specific granulation tissue on day 7 and evidence of localized bacterial infection on day 9. The histology of the allogeneic skin graft appeared normal on day 0, showed slight vacuolization on day 4, developed a dense cellular infiltrate by day 7 and appeared to be fully rejected by day 9, with histologic evidence of a regenerating host skin bed beneath the rejected graft. Consistent with the gross observation of a white graft, the Gal-positive pig skin never showed histologic evidence of engraftment (Fig. 2). Histology on day 4 showed thrombi in small vessels, consistent with hyperacute rejection, leading to occlusion of the blood vessels. Immunohistochemistry revealed a large amount of anti-Gal but not anti-non-Gal antibody deposition by this time (Fig. 4A,B), and the graft was necrotic by day 7. In contrast, the GalT-KO skin graft appeared essentially normal on days 4 and 7, with only mild congestion. Antibody deposition was not observed by immunohistochemistry until day 9, at which point the graft showed

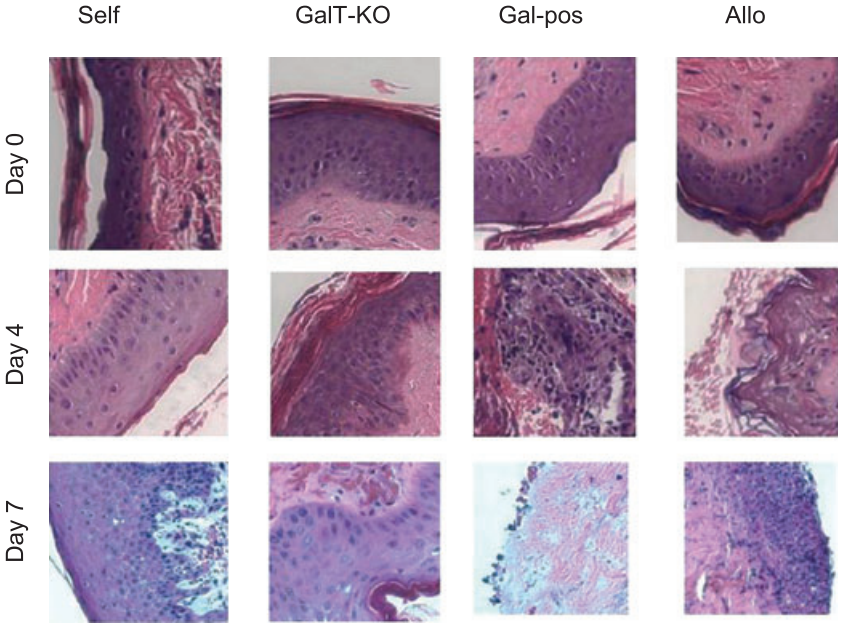

Fig. 2. Histology of skin grafts on baboon 1, biopsies on days 0, 4, and 7.

evidence for cellular rejection similar to that seen in the allograft on day 7 (data not shown).

In baboon 2, the interpretation of early histologic samples of skin graft biopsies was less clear. However, by day 4, the Gal-positive skin showed antibody deposition, and by day 11 it appeared completely non-viable, respectively. In contrast, in the GalT-KO skin graft, antibody deposition was observed only after day 7 and the graft remained viable on day 11 (Fig. 4C,D). It still showed partial viability on day 14, although with considerable inflammation. The graft bed was visible in this sample, confirming that the viable epidermis was in fact graft-derived.

Antibody responses to skin grafts

Cytotoxicity assays with and without DTT showed that despite high levels of anti-Gal and anti-non-Gal preformed IgG in both baboons, all antibody-mediated cytotoxicity in the first week was almost entirely mediated by preformed anti-Gal IgM. After 7 days, however, both the amount and cytotoxicity of anti-Gal and anti-non-Gal IgG increased markedly (Fig. 3).

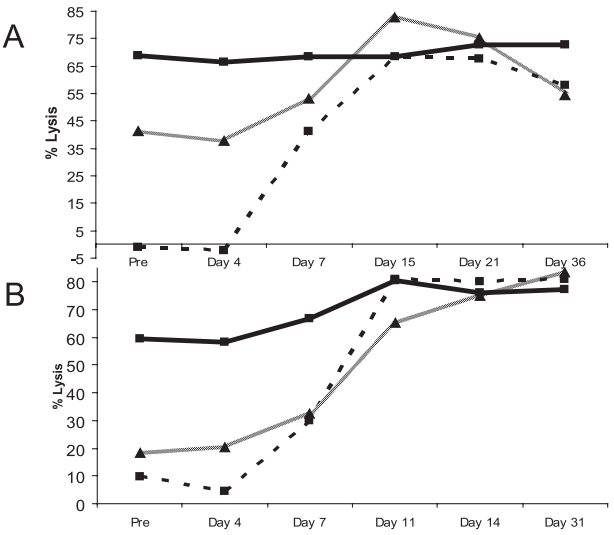

Fig. 3. Antibody-complement mediated cytotoxicity assay of sera from baboons 1 (A) and 2 (B) at 1 : 4 dilution showed increased antibody-mediated cytotoxicity after day 4, with higher baseline killing levels against Gal-positive cells than against GalT-KO cells. Addition of DTT demonstrated that early cytotoxicity was largely due to preformed anti-Gal IgM (solid black line ■--■ = Gal-positive cell targets without DTT, solid gray line ▲--▲ = GalT-KO cell targets without DTT, dashed black line = Gal-positive cell targets with DTT).

Discussion

The results reported in this preliminary study suggest that GalT-KO xenografted skin is preferable to normal, Gal-positive swine skin, and as good as or better than allogeneic skin (i.e., cadaveric human skin) for use as an acute skin transplant to cover severe burn injuries. The gross observations on the skin grafts in this study were substantiated by the histological findings in both animals.

The antigen most responsible for rapid rejection of normal pig tissues is the α-1,3-Galactose (Gal) moiety, found on the cell surfaces of all mammalian species, except Old World primates and humans [15]. Primates have high levels of preformed antibodies to Gal and after transplantation, these antibodies bind to the Gal antigen on

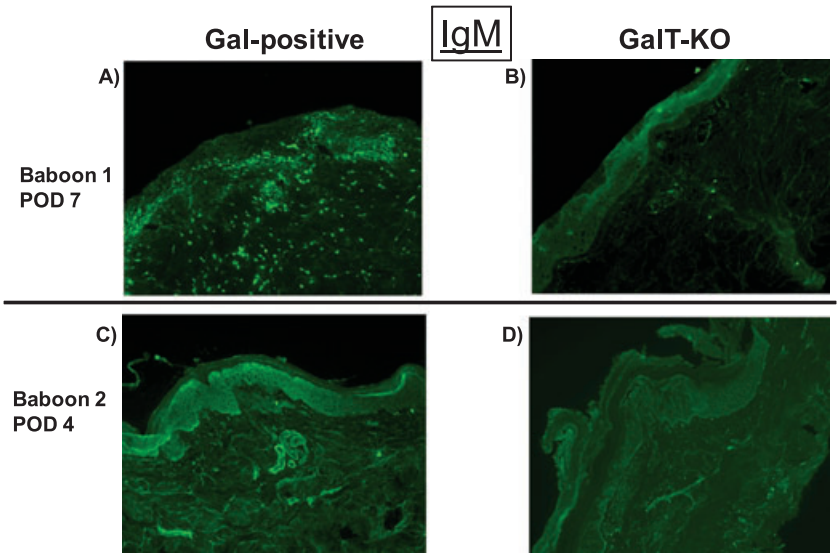

Fig. 4. Immunohistochemistry of Gal-positive (A,C) and GalT-KO (B,D) skin on baboons 1 (POD 7) and 2 (POD4) showing early deposition of anti-Gal IgM but not anti-non-Gal IgM.

vascular endothelial cells. There, they fix complement, damaging the endothelium and triggering the coagulation cascade, resulting in immediate rejection by occlusion of graft vessels [25]. This process, which is dependent on both antibody levels and on the level of antigen expression on endothelial cells [26], was likely responsible for the early rejection of Gal-positive skin in both baboon recipients in the present study, leading to non-vascularized or "white grafts" by day 4 in both cases. The much lower levels of preformed anti-non-Gal antibodies were likely responsible for the much milder evidence of humoral rejection observed several days later for GalT-KO skin. Although some humans also have antibodies against non-Gal pig antigens, previous studies in our laboratory have demonstrated that these antibodies are generally of low prevalence and titer [22].

Therefore, prolonged survival of GalT-KO skin compared with Gal-positive skin confirms the importance of immune recognition of Gal. However, it is less clear why the survival of GalT-KO skin should have been prolonged over that of allogeneic skin, as in neither case would high levels of anti-donor antibody-mediated reactivity be expected. Preliminary mixed lymphocyte reaction data indicate that, in the absence of a major contribution of humoral rejection, the strength of anti-donor cellular responses may determine the kinetics of rejection (data not shown). Further experiments are in progress to determine whether or not this correlation is generalizable and will be reported in a subsequent publication.

In summary, these results suggest that xenogeneic pig skin from GalT-KO swine may provide a less expensive, more readily available and potentially long-lasting alternative to allogeneic human skin as an initial covering for extensive burn injuries.

Acknowledgments

This work was supported in part by grants from the Department of Defense (Grant Number: DR080729) and the NIH/NIAID (Grant Number: 5P01AI45897-09).
Patents: Alpha (1,3) Galactosyltransferase Negative (GalT-KO) Swine (00841.10)

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Gal-KO Xenoskin Graft Survival is Comparable to Skin Allotransplantation for Burn Injury

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INTRODUCTION: Efficacious, readily-available, cost-effective therapies are lacking for severely burned patients. Xenografting may provide patients with temporary coverage. Gal-KO swine lack the α-1,3-galactosyltransferase gene, associated with hyperacute rejection across xenogenic barriers. Gal-KO skin may provide coverage of burns, , analogous to currently-used cadaveric allografts.

METHODS: Non-Human Primates underwent split-thickness skin transplantation. Baboon 1 received self, allo-baboon, Gal-KO, and Gal+ xenografts. Baboon 2 underwent the same procedure but received immunosuppressive therapy with CyA (15 mg/kg/day). Grafts were visualized and punch-biopsied on PODs 4,7,11,14,21 and 30. Following rejection of the primary grafts and presumed sensitization, animals were re-transplanted on POD41 with self, allo, fresh Gal-KO skin and frozen Gal-KO skin. CyA levels were measured q3d, maintaining therapeutic circulating levels of 200-600 ng/mL. Mixed Lymphocyte Reaction (MLR), ELISPOT, and flow cytometric immunologic assays were performed at multiple time points prior to and following transplant.

RESULTS: In both animals, Gal-KO xenografts and baboon allografts were viable on POD11 and rejected by POD14. Gal+ xenografts displayed hyperacute rejection by POD4 in both animals. No clinical difference in graft survival was observed between the two animals (with and without CyA). Gal-KO retransplants were rejected by POD4. No difference was observed between fresh or frozen xenografts. Pathology confirmed clinical rejection in all cases. Sensitization was confirmed using MLR. Class switching of anti-nonGal IgM to IgG was observed on FACS and ELISPOT.

CONCLUSIONS: Gal-KO xenografts are comparable to allografts. Sensitization leads to hyperacute rejection. Hyperacute rejection of Gal+ skin occurs by POD4 due to anti-Gal Abs. The lack of a significant difference in viability between fresh and frozen GalT-KO skin suggests that this model could be used for treatment of severe burns when cadaver skin is not readily available.

Comparable Graft Survival of GalT-KO Pig Skin and Allogeneic Skin on Baboons

Angelo A Leto Barone[1, 2], Joshua Weiner[1], John S Hanekamp[1], Kazuhiko Yamada[1], Curtis L Cetrulo, Jr [1, 2] and David H Sachs[1]

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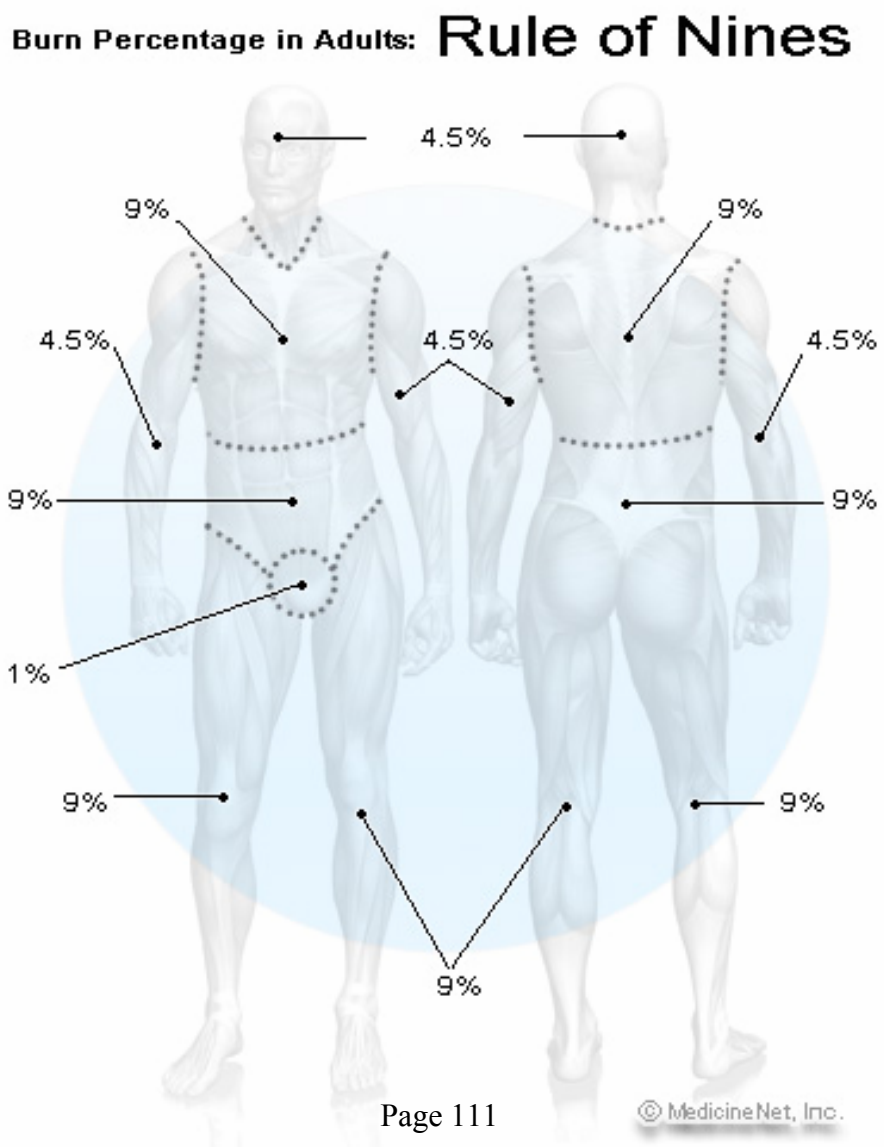
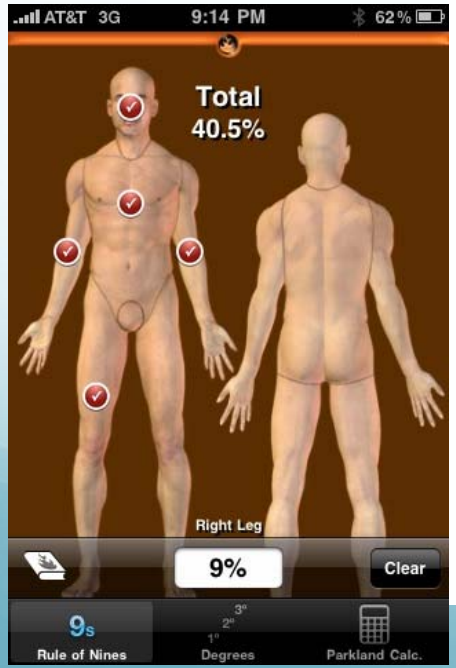
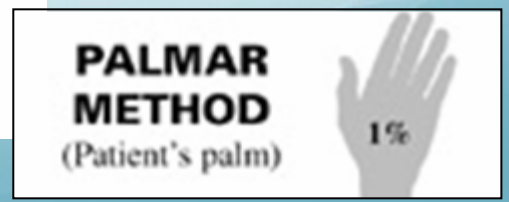
Massachusetts General Hospital, Harvard Medical School, Boston, MA

The Need

Burn Assessment

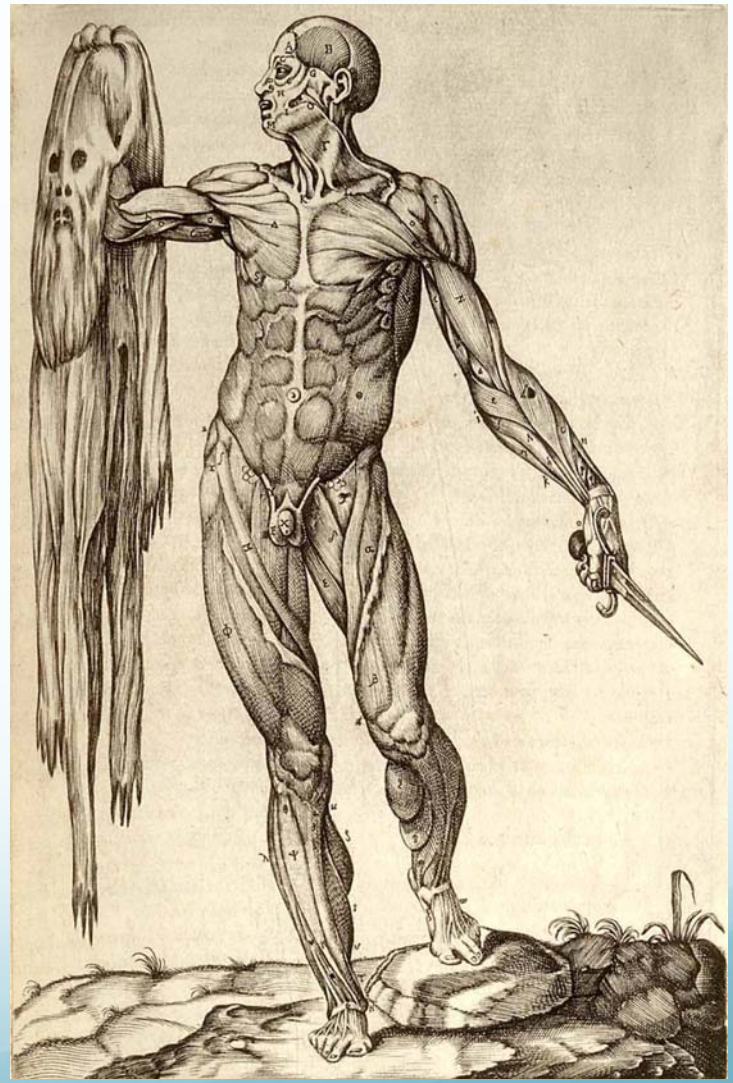






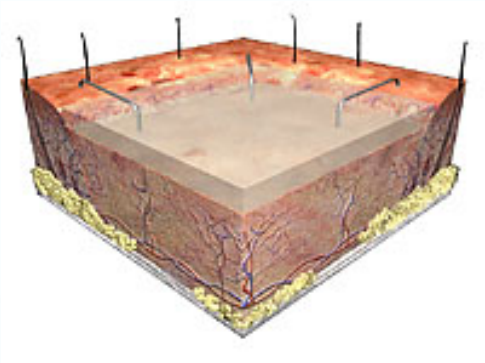
Main Goals of Burn Treatment:

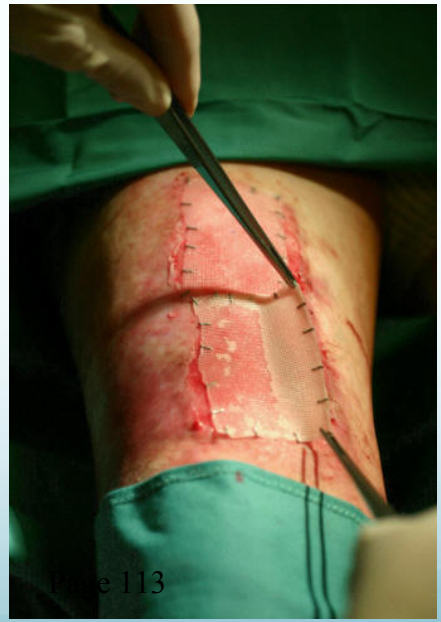
- **Maintain Barrier function**
  - **Retain Fluids**
  - **Preserve Body Temperature**
- **Minimize Infection**



Skin Replacement options

- Autografts
- Allografts from cadaveric skin
- Skin expansion
- Biomaterials (Integra®)
- Keratinocytes
- Glutaraldehyde-preserved Swine Skin



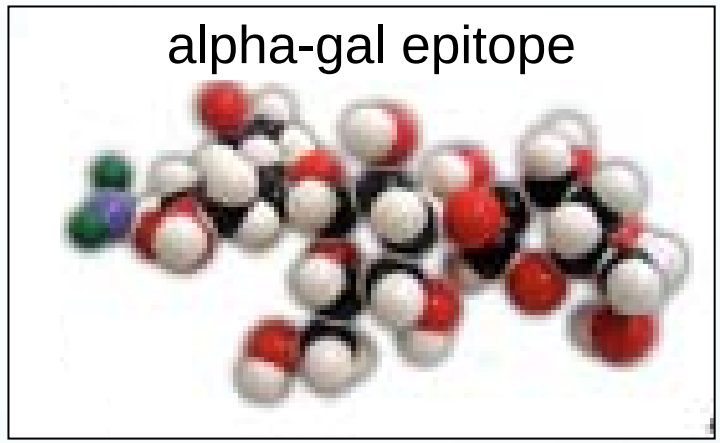


Background

- Galactose-α1,3-galactose epitope (Gal) is ubiquitously expressed in pig cells

- Gal is associated with hyperacute rejection of organs across xenogeneic barriers

- Hyperacute rejection is due to natural preformed anti-Gal antibodies present in primates



GalT-KO Xenografts

- 2002: Development of α-1,3-galactosyltransferase KO (GalT-KO) swine
  - Lacks Gal epitope
  - Circumvents hyperacute rejection of organs by primates

- Question: Could GalT-KO skin provide temporary early coverage of burns, analogous to currently-used cadaveric *allo*grafts?

“Goldie”, November 18, 2002

Genetically-Modified Porcine Skin Grafts for Treatment of Severe Burn Injuries

- Aim 1: To assess whether GalT-KO skin provides coverage for as long as Allo skin

  - Immunosuppression vs. No Immunosupression **(CyA vs. no CyA)**

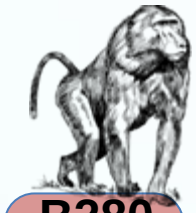
- Aim 2: To compare Fresh vs Frozen skin in primary and secondary grafts

- Aim 3: To investigate the sensitization process following Xeno and Allo skin transplantation

Aim 1

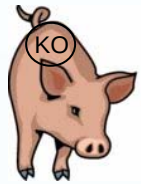
XENO SKIN GRAFTS

Donors

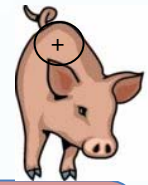


B280
Self

B282
Allo



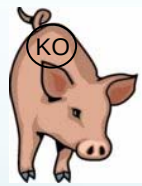
GalT-KO



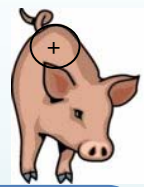
GalT +

B282
Self

B280
Allo



GalT-KO



GalT +

Recipients

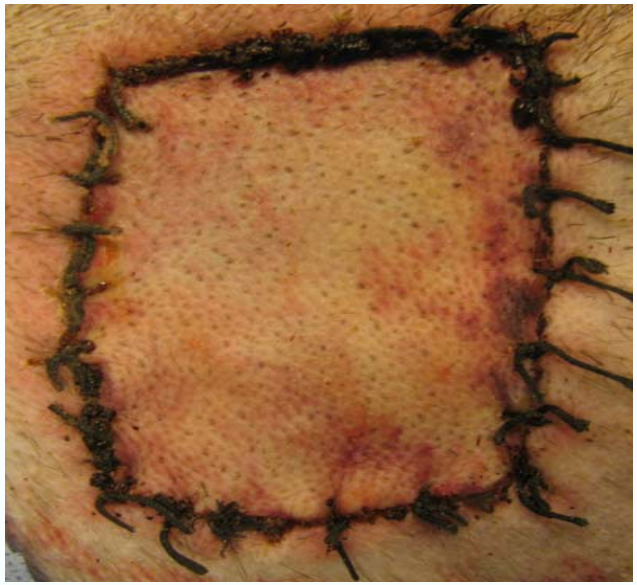
B280

B282

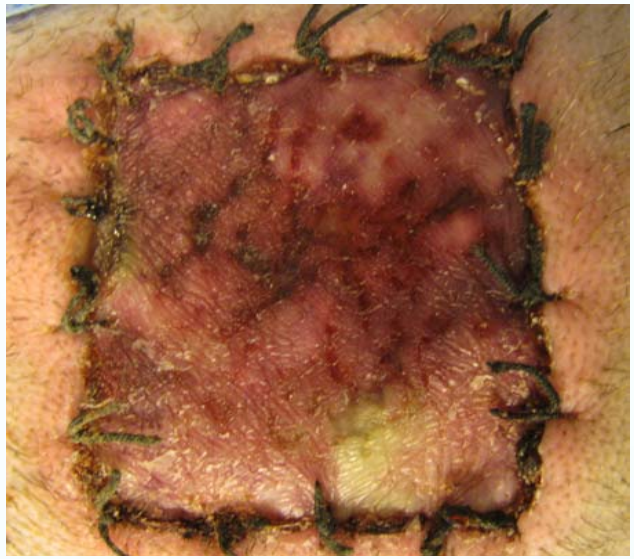
(CyA)

B280 (No CyA)

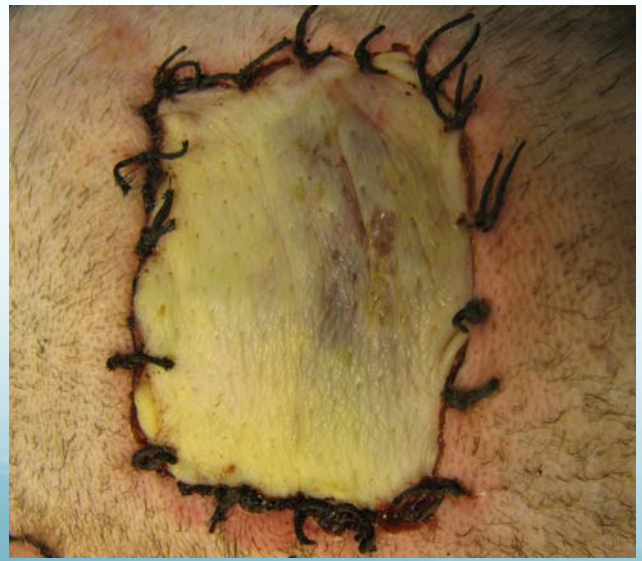
Self



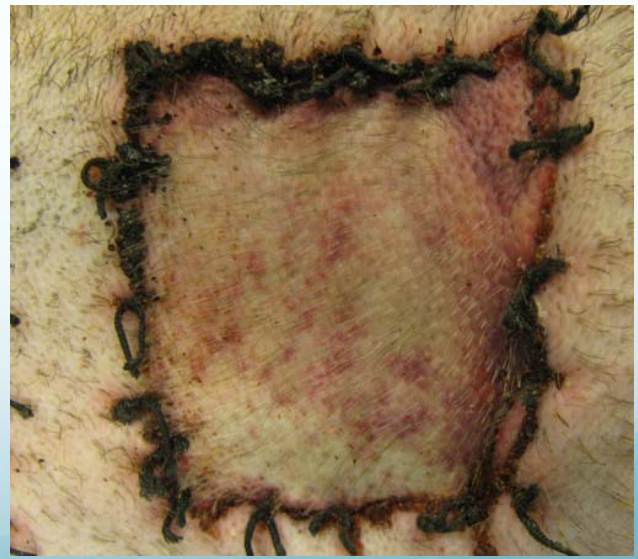
Gal KO



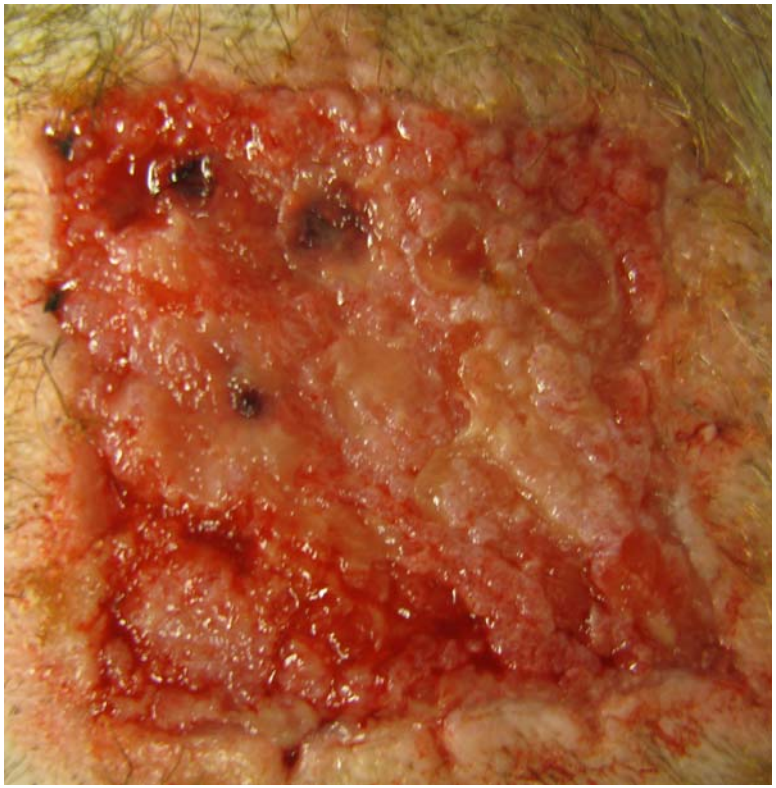
POD4



Gal +



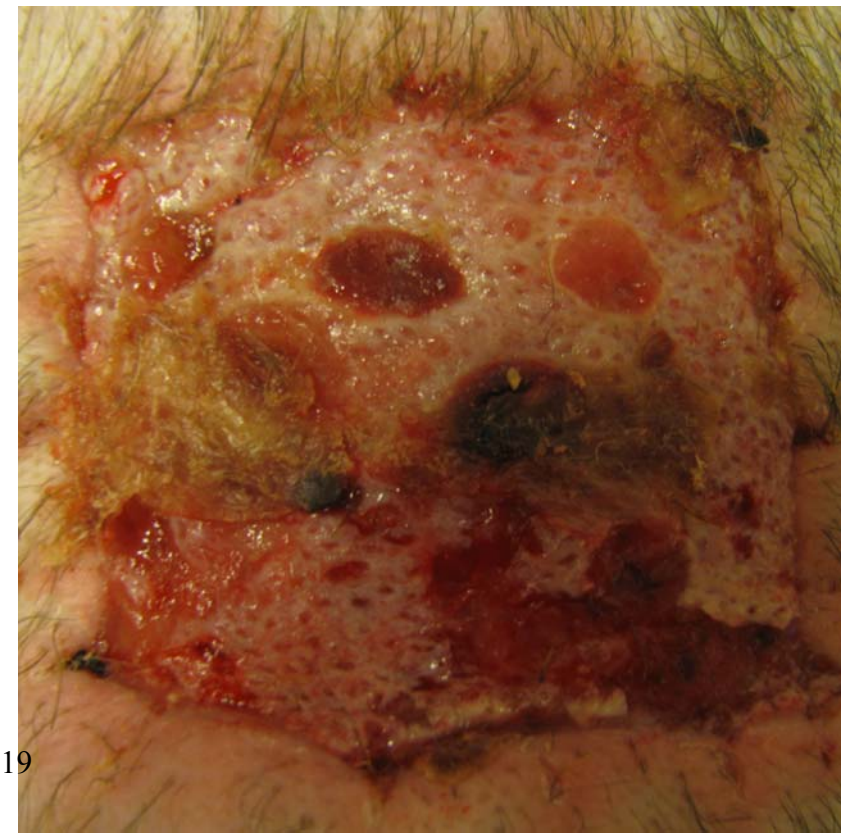
Allo



B280 (No CyA)

POD13

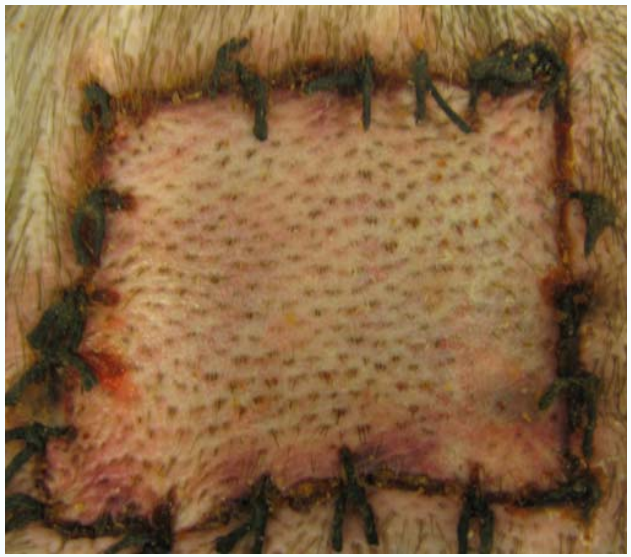
Allo



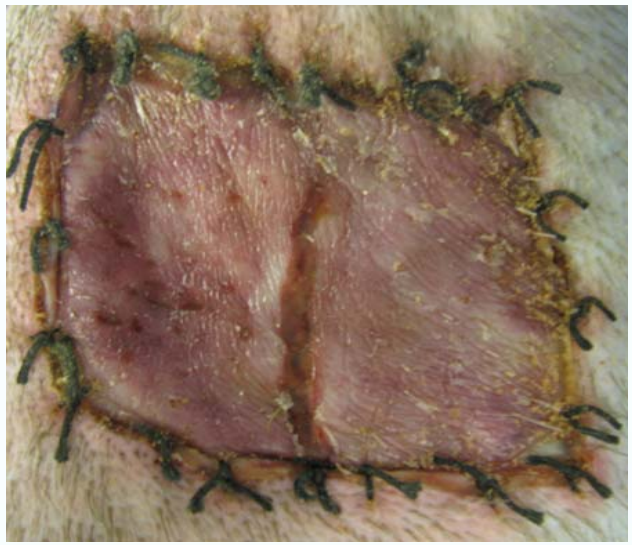
Gal KO

B282 (CyA)

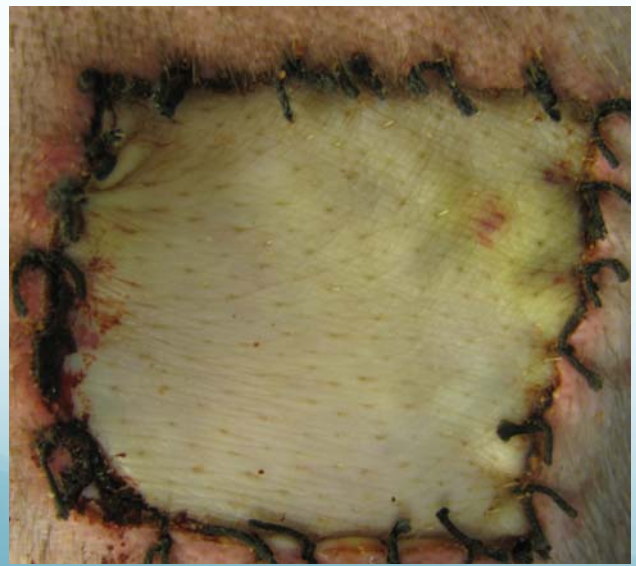
Self



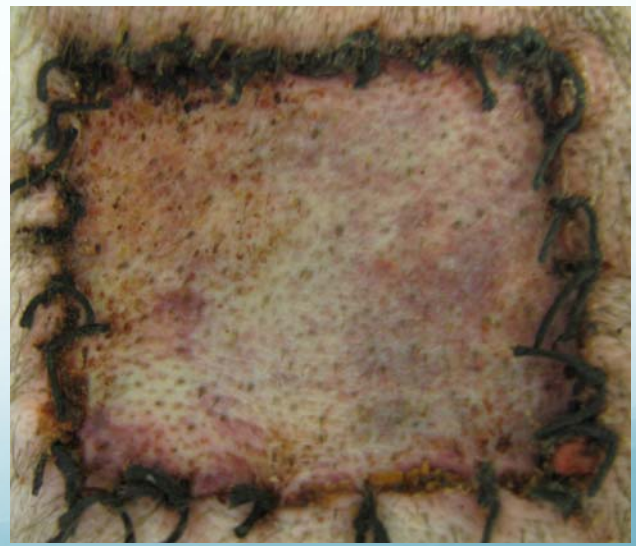
Gal KO



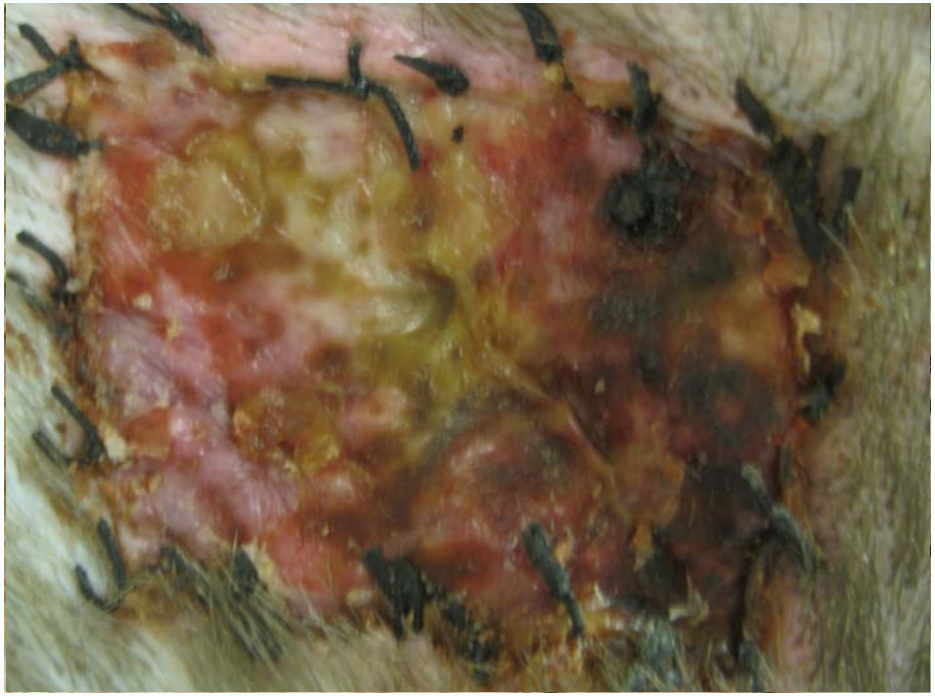
POD4



Gal +



Allo

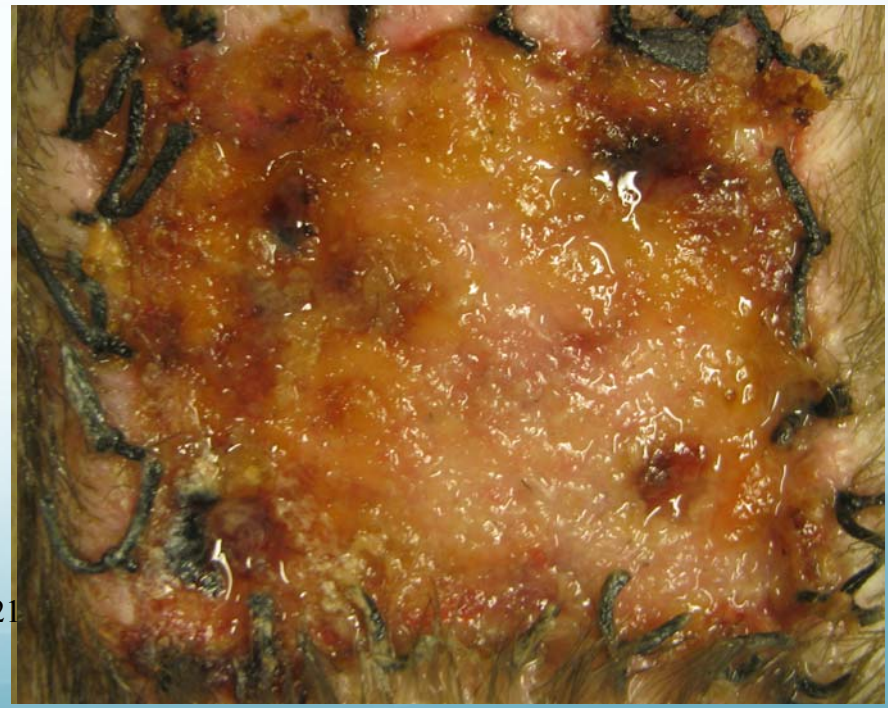


Gal KO

B282 (CyA)

POD11

Allo



Genetically-Modified Porcine Skin Grafts for Treatment of Severe Burn Injuries

- Aim 1: To assess whether GalT-KO skin provides coverage for as long as Allo skin

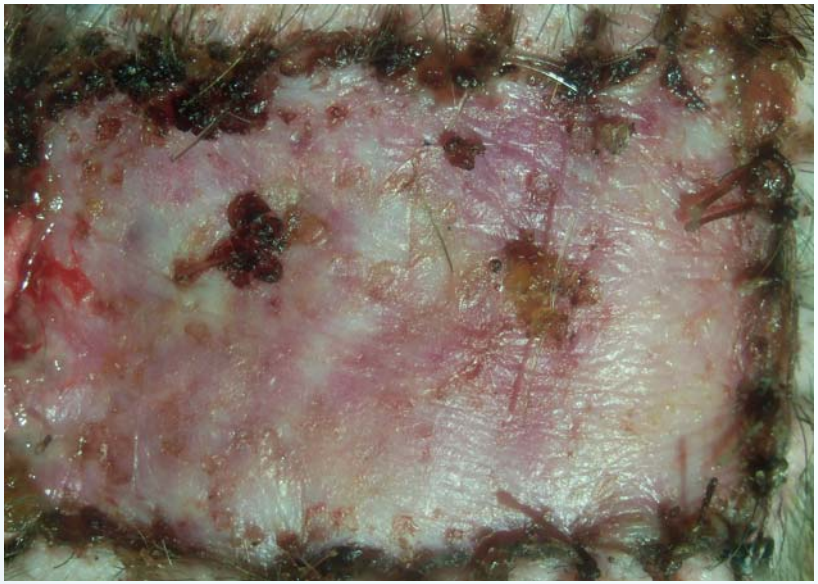
  - Immunosuppression vs. No Immunosupression **(CyA vs. no CyA)**

Conclusions:

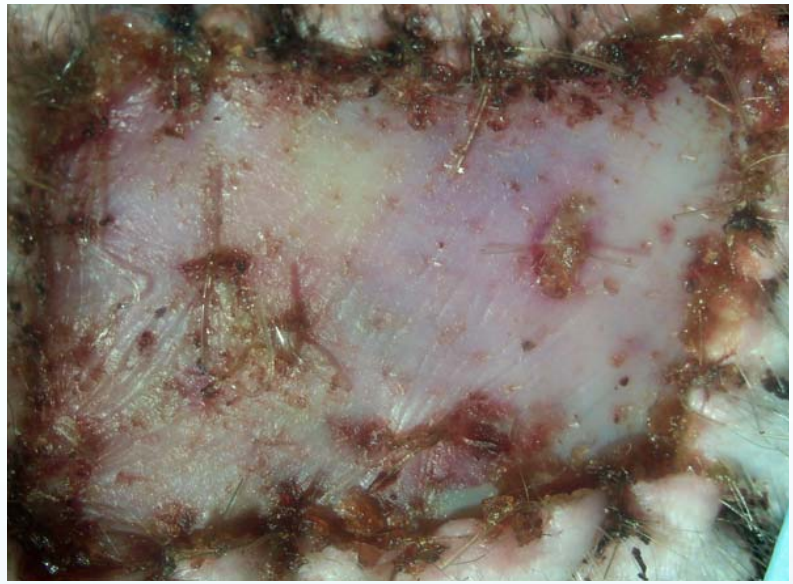
- **GalT-KO skin provides temporary coverage comparable to Allogeneic skin**
- **CyA prolonged survival of skin grafts by 1-2 days**

Aim 2: To Compare Fresh vs. Frozen GalT-KO Skin in Primary Grafts

Split -Thickness Skin Grafts over full thickness wounds

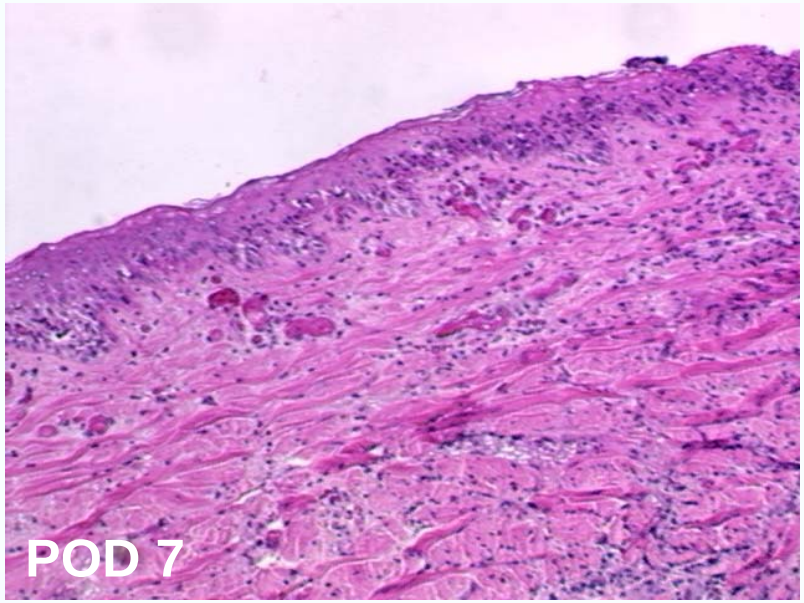


Gal KO Fresh

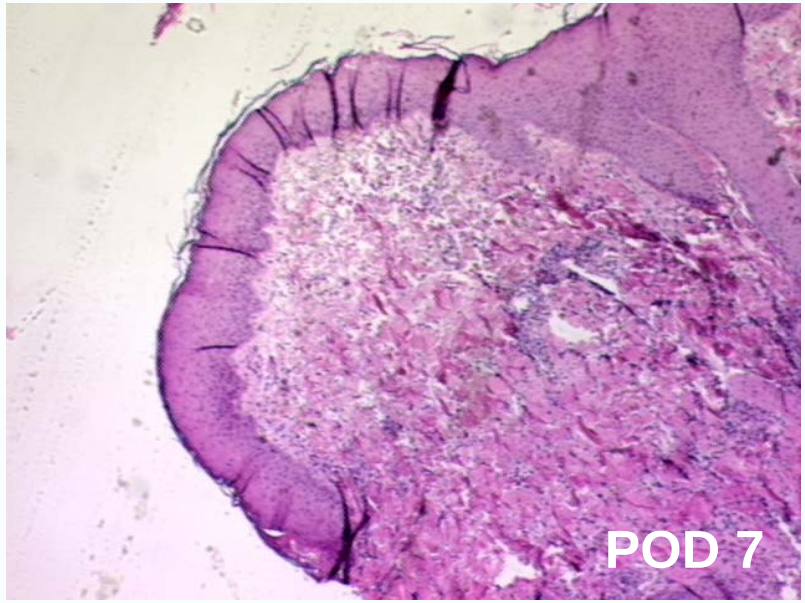


Gal KO Frozen

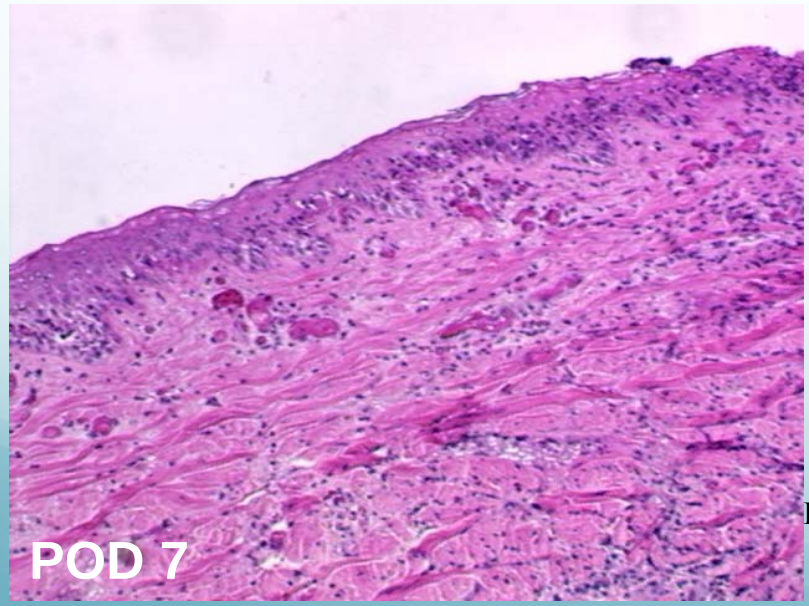
GalT-KO (no CyA)



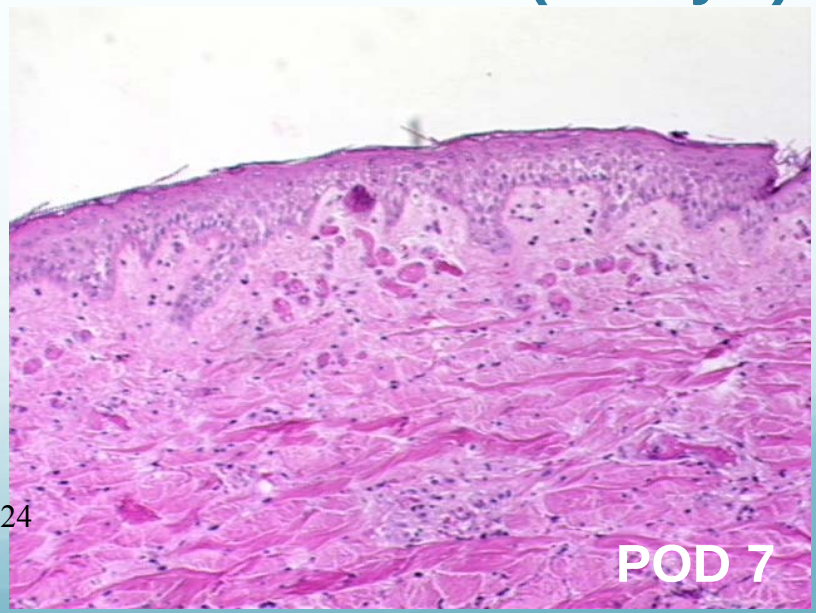
Allo skin (no CyA)



GalT-KO Fresh (no CyA)



GalT-KO Frozen (no CyA)



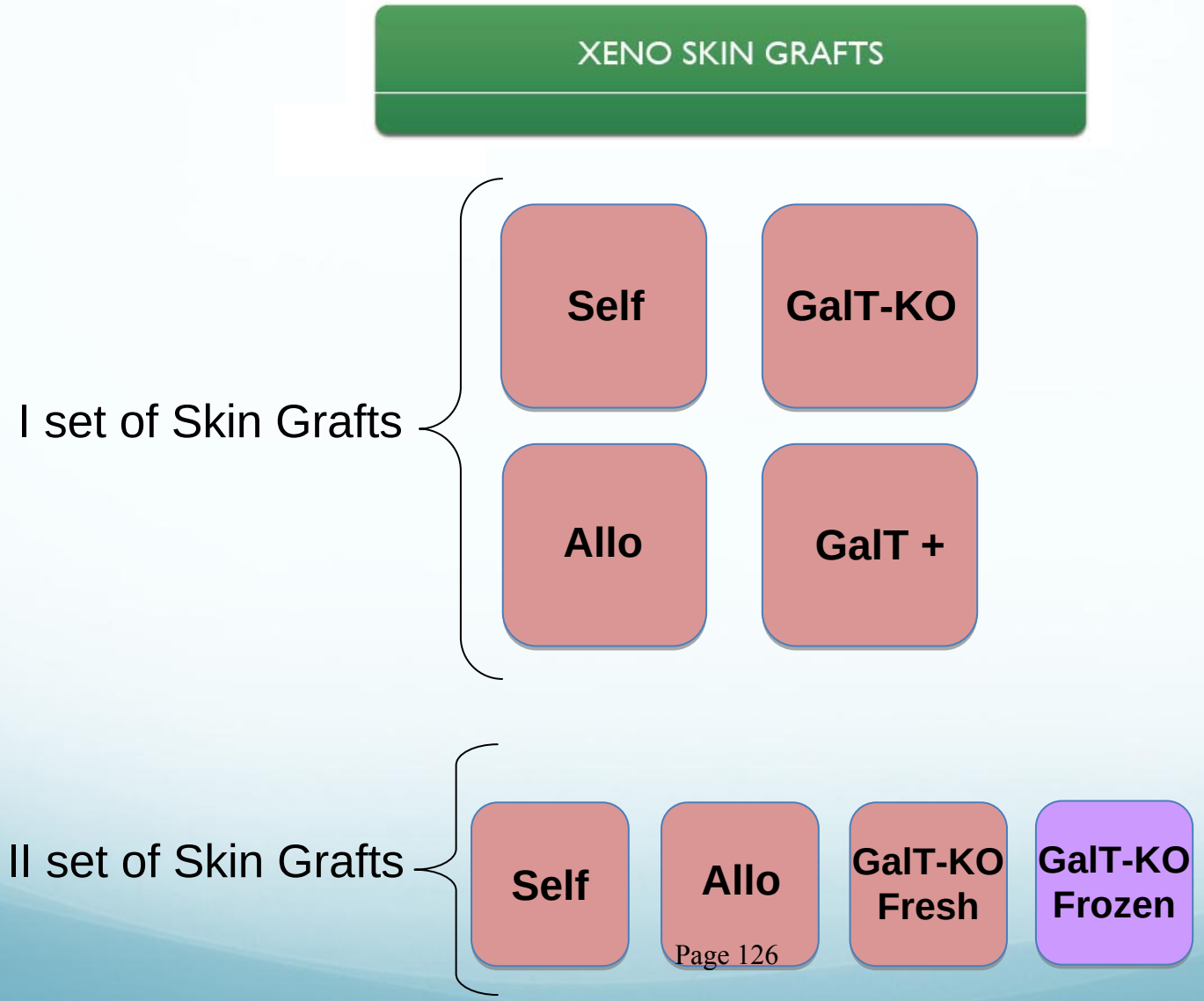
Genetically-Modified Porcine Skin Grafts for Treatment of Severe Burn Injuries

- Aim 2: To compare Fresh vs Frozen skin in primary and secondary grafts

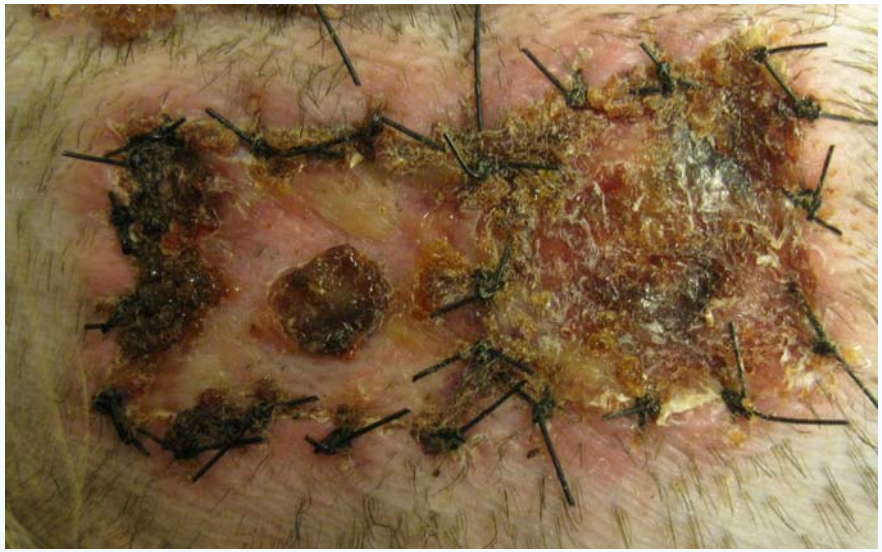
Conclusions:

- No major differences were observed between fresh and frozen GalT-KO skin

Aim 3: To investigate the sensitization process following Xeno and Allo skin transplantation

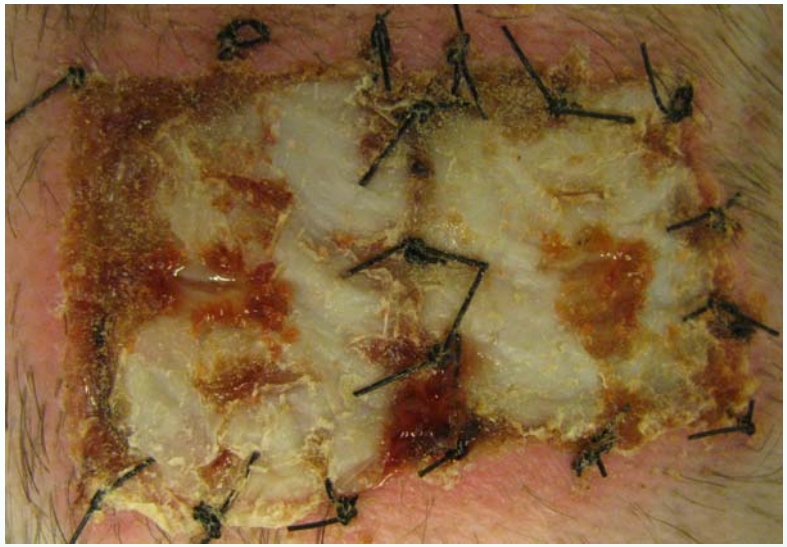


GalT-KO skin regrafts rejected hyperacutely in sensitized animals



Self

Allo



KO fresh

KO frozen

POD4

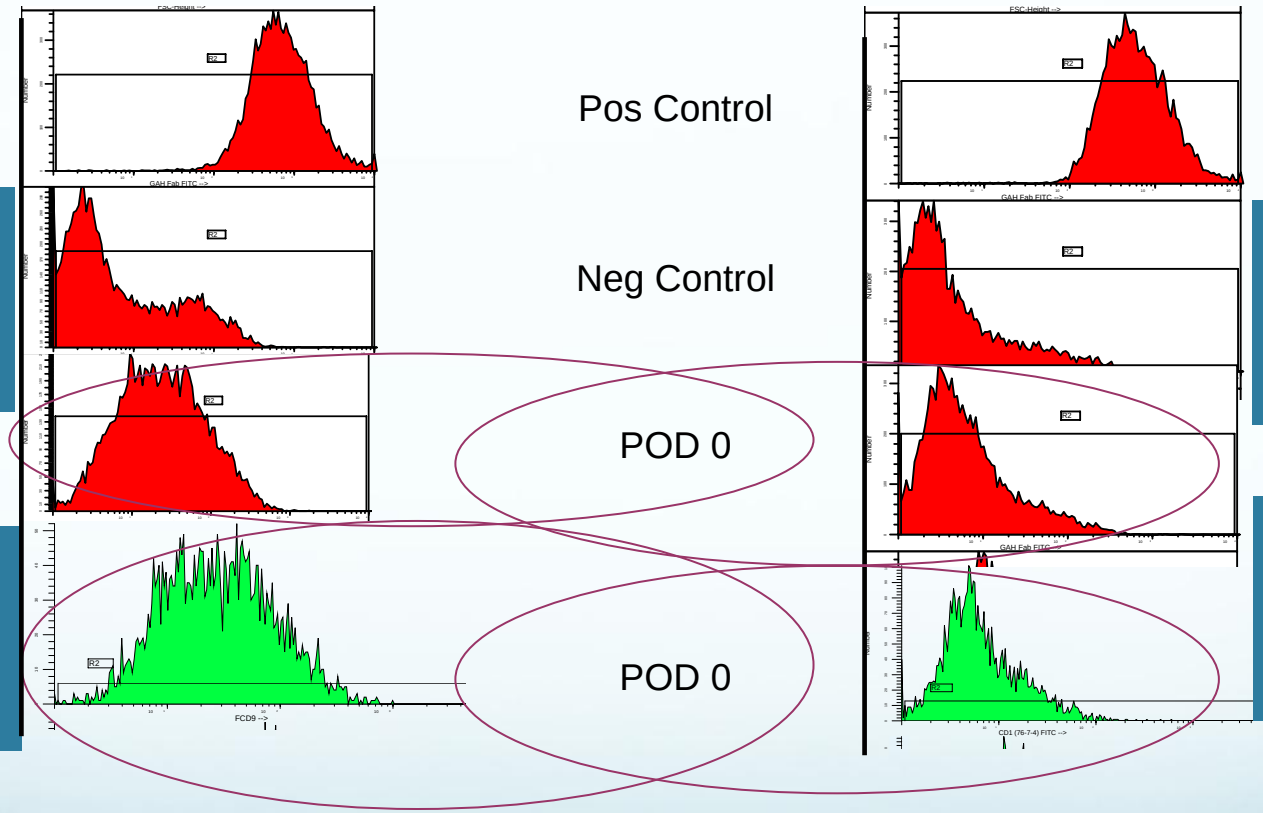
No CyA

Lack of Anti-non-Gal Ab before TX

B266

Anti-Gal IgM FACS (DD gal+ cells)

Anti-Gal IgG FACS (DD gal+ cells)

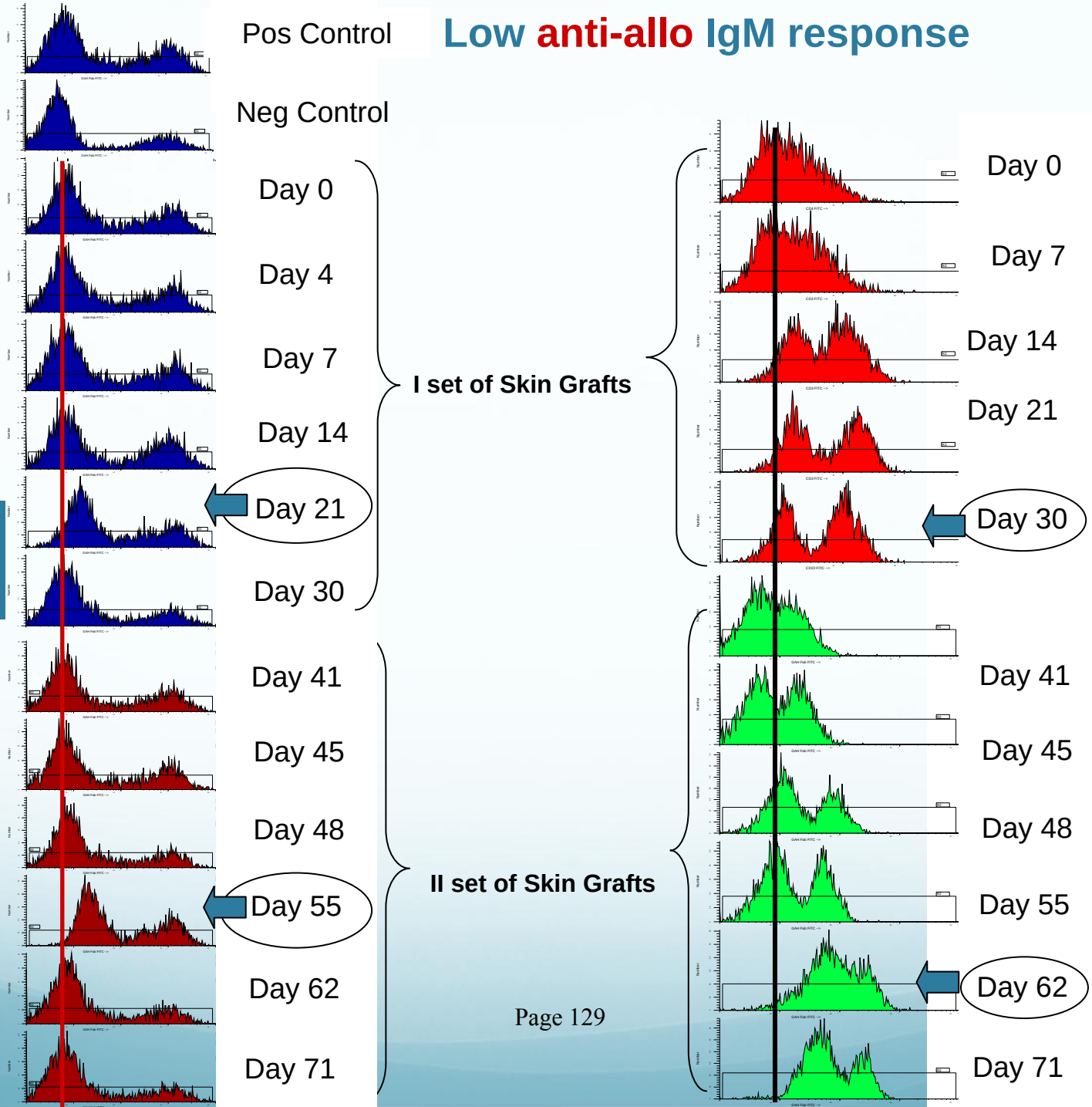


Anti-non-Gal IgM FACS (Gal KO cells)

Anti-non-Gal IgG FACS (DD gal+cells)

Low anti-allo IgM response

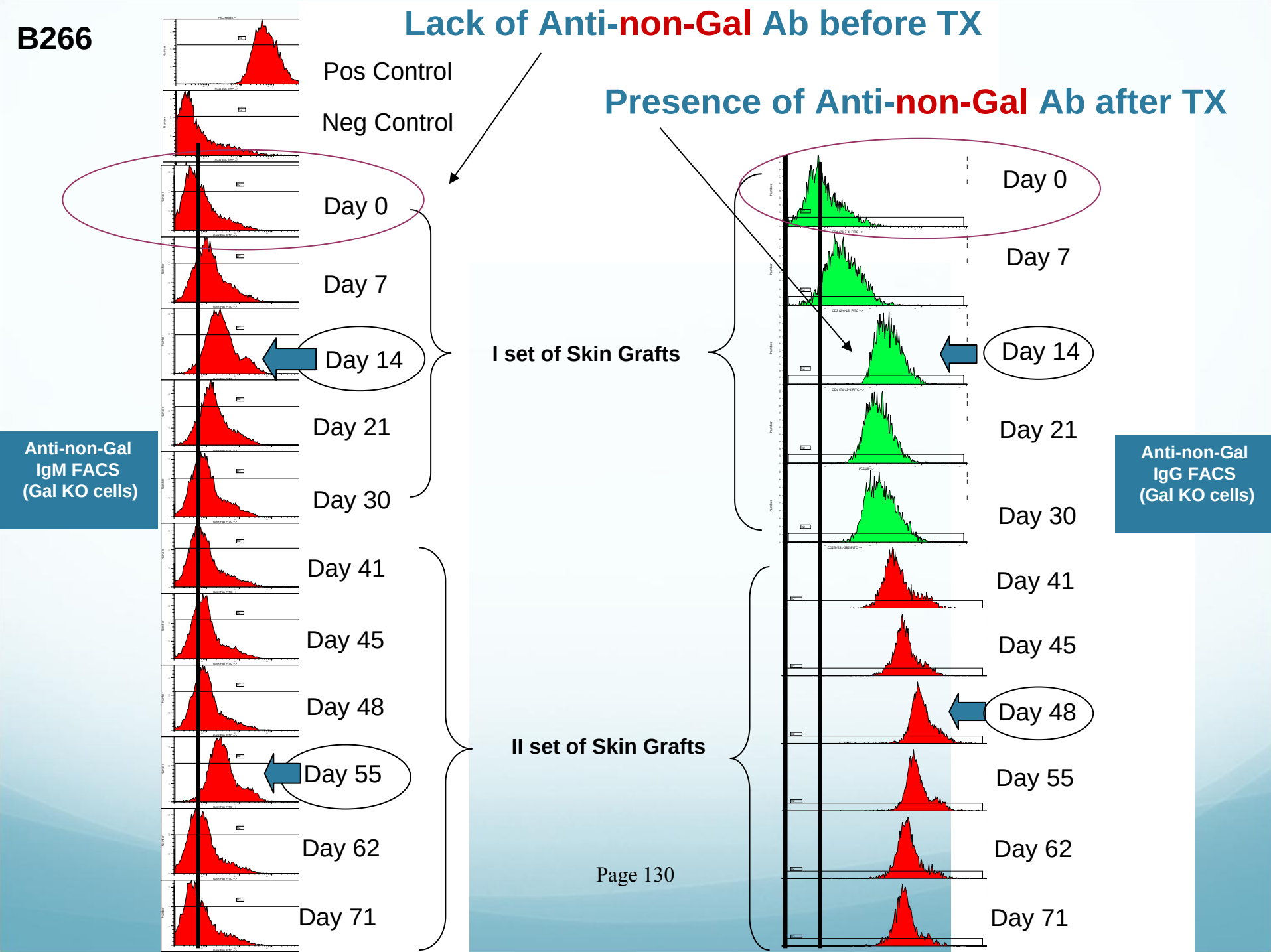
B266



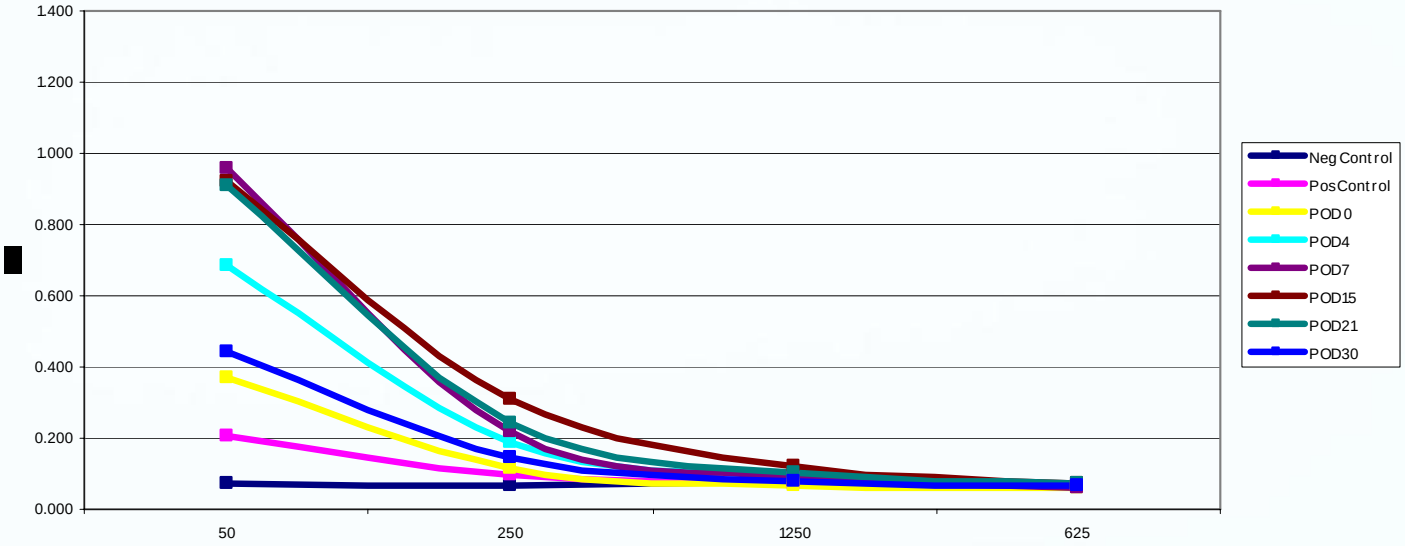
Anti-Allo IgM FACS

Anti-Allo IgG FACS

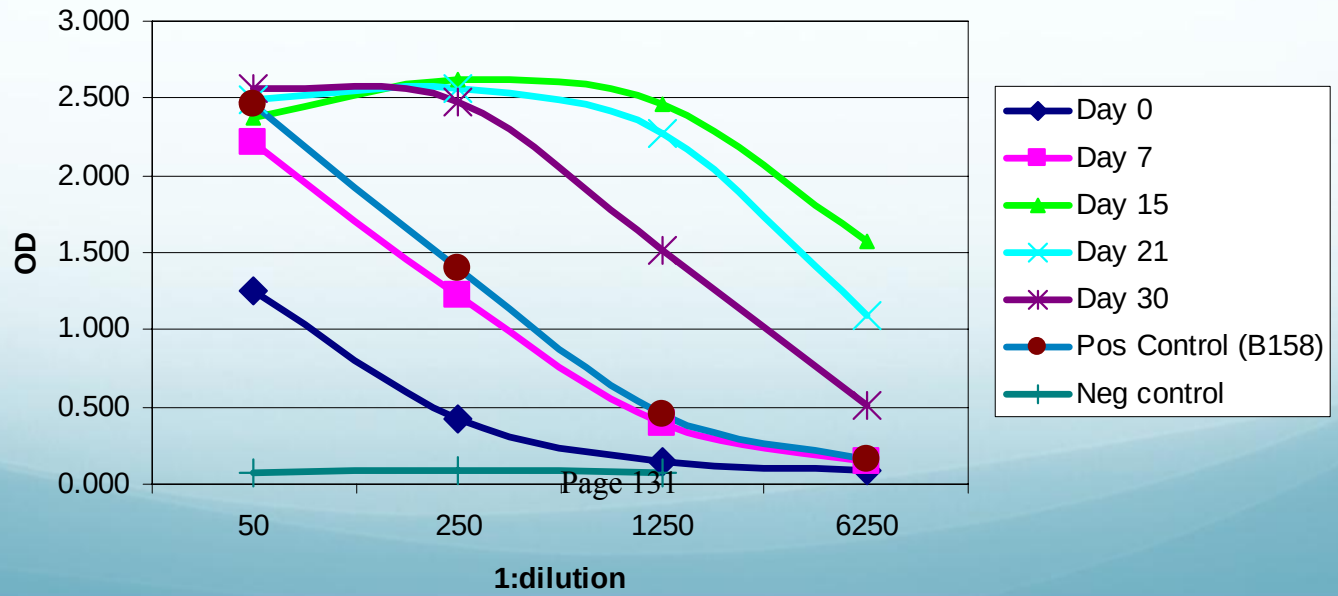
B266


Lack of Anti-non-Gal Ab before TX
Presence of Anti-non-Gal Ab after TX
Pos Control
Neg Control
Day 0
Day 7
Day 14
Day 21
Day 30
Day 41
Day 45
Day 48
Day 55
Day 62
Day 71
I set of Skin Grafts
II set of Skin Grafts
Anti-non-Gal IgM FACS (Gal KO cells)
Anti-non-Gal IgG FACS (Gal KO cells)

B266 Anti-Gal IgM ELISA (s/p 1st set of grafts)

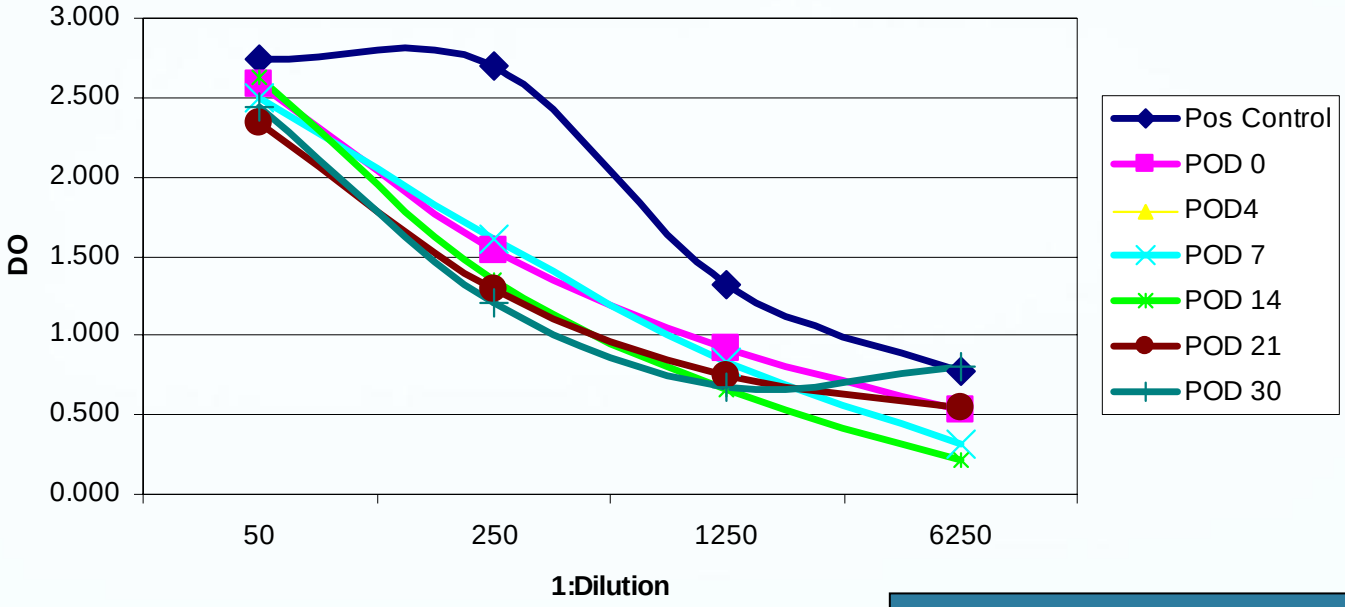


B266 Anti-Gal IgG ELISA (s/p 1st set of grafts)



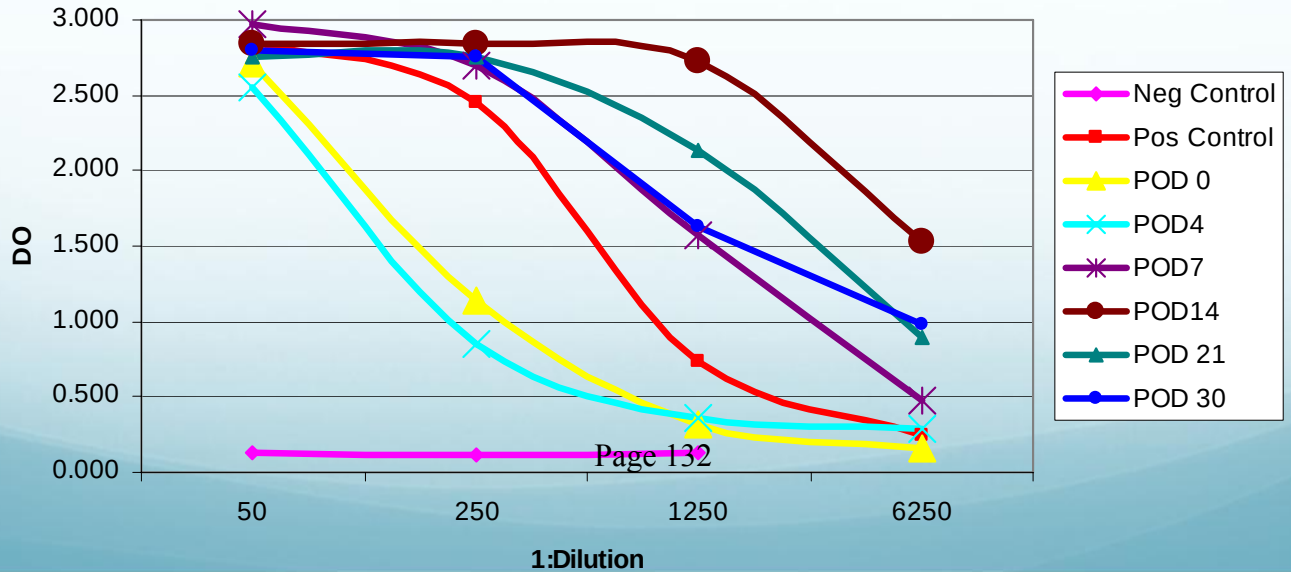
B268 Anti-Gal IgG ELISA

GalT-KO skin graft only



B269 Anti-Gal IgG ELISA

GalT + skin graft only



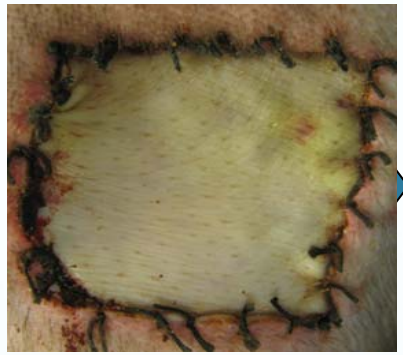
Experimental Observations

1st graft

2nd graft

GalT +

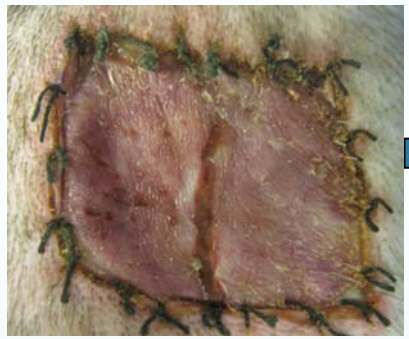
Preformed Ab Response



White Graft

GalT-KO

No preformed Ab

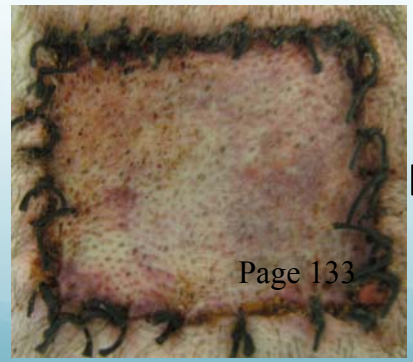


Sensitization

Post regrafting

Allo

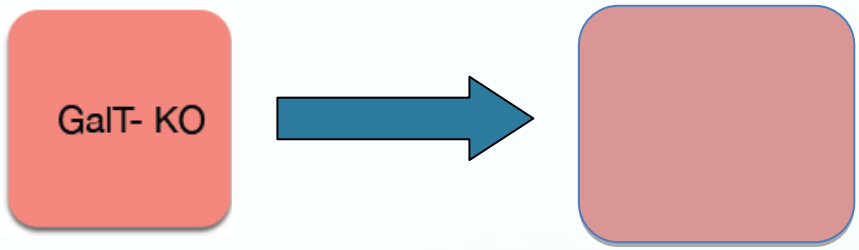
No preformed Ab



Sensitization

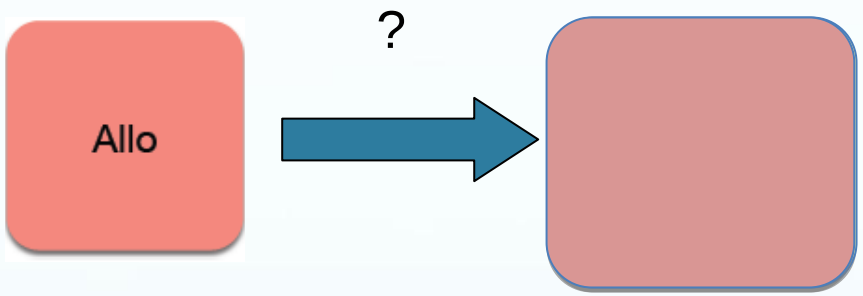
Possible Clinical Treatment Strategy (currently being studied)

1st Line of Treatment

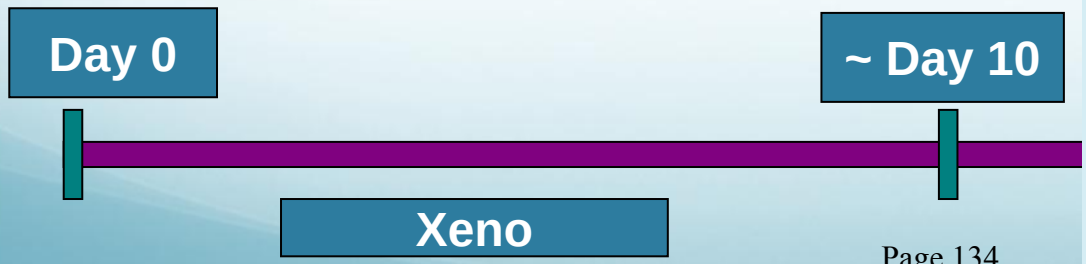


Burn site coverage

2nd Line of Treatment



Burn site coverage



Summary

- GalT-KO skin xenografts exhibit comparable survival to allografts (Xenografts = Allografts)

- GalT-positive skin is rejected in a Hyperacute fashion, presumably due to preformed antibodies (White Graft)

- No differences in survival of fresh vs frozen GalT-KO skin was observed following re-transplant

Summary

- GalT-KO skin can be used as a temporary skin substitute in severe burns in lieu of Allografts or Biomaterials

- Current studies: Can Xeno skin be used as a first line skin substitute to allow for subsequent successful use of Allografts and prolong wound coverage?

Acknowledgments

Transplantation Biology Research Center (TBRC)

- Curtis L. Cetrulo Jr, M.D.
- Radbeh Torabi
- Fan Liang, M.D.
- David H. Sachs, M.D.

- TBRC Staff

Harvard Plastic Surgery

- Jay Austen, M.D.
- Michael Yaremchuk, M.D.
- Mark Randolph, M.S.

DOD Grant: W81XWH-09-1-0419

MASSACHUSETTS GENERAL HOSPITAL

Subcommittee on Research Animal Care
Massachusetts General Hospital
149 13th Street
Charlestown, MA 02129
Tel: (617) 726-3495
Fax: (617) 724-2475

Notification of IACUC Review

Protocol #: 2009N000062 / 6

Date: March 31, 2010

To: David H. Sachs, MD
MGH, Surgery
CNY 149 9019

From: Diane McCabe, IACUC Manager
Research Management

Title of Protocol:	Genetically-modified Porcine Skin Grafts for Treatment of Severe Burn Injuries (Swine)
Sponsor:	ARMY-DOD
Species / Number:	Swine / 264
Annual Review #:	1
Approval Date:	03/31/2010
Expiration Date:	05/20/2012
Annual Report Due:	03/31/2011

This Annual Report has been reviewed and approved by the MGH Subcommittee on Research Animal Care (SRAC) – OLAW Assurance # A3596-01. The protocol as submitted and reviewed conforms to the USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, the "ILAR Guide for the Care and Use of Laboratory Animals" and other applicable laws and regulations. The protocol is approved for a three-year period, subject to submission of annual reports.

Please note that if a SRAC member had a conflict of interest with regard to the review of this protocol, that member left the room during the discussion and the vote on this project.

As Principal Investigator you are responsible for the following:
1. Compliance with MGH SRAC/CCM Policies governing the care and use of animals.
2. Submission in writing of changes to this project for SRAC review and approval prior to initiation of the change.
3. Submission of annual progress reports to the SRAC for review and approval.
4. Submission of a copy of this approval to CCM when ordering animals.

The SRAC can and will terminate projects that are not in compliance with these requirements. Direct questions, correspondence and forms to Diane McCabe, the IACUC Manager, Tel: (617) 724-9718, Fax: (617) 724-2475.

MASSACHUSETTS GENERAL HOSPITAL

Subcommittee on Research Animal Care
Massachusetts General Hospital
149 13th Street
Charlestown, MA 02129
Tel: (617) 726-3495
Fax: (617) 724-2475

Notification of IACUC Review

Protocol #: 2009N000063 / 1

Date: August 25, 2009

To: David H. Sachs, MD
MGH, Surgery
CNY 149 9019

From: Diane McCabe, IACUC Manager
Rese arch Management

Title of Protocol:	Genetically-modified Porcine Skin Grafts for Treatment of Severe Burn Injuries (Baboons)
Sponsor: ARMY-DOD	
Species / Number:	Monkeys - Baboon / 137
Approval Date:	07/15/2009
Effective Date:	08/25/2009
Expiration Date:	07/15/2012
Annual Report Due:	07/15/2010

This Protocol has been reviewed and approved by the MGH Subcommittee on Research Animal Care (SRAC) – OLAW Assurance # A3596-01. The protocol as submitted and reviewed conforms to the USDA Animal Welfare Act, PHS Policy on Humane Care and Use of Laboratory Animals, the "ILAR Guide for the Care and Use of Laboratory Animals" and other applicable laws and regulations. The protocol is approved for a three-year period, subject to submission of annual reports.

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MASSACHUSETTS GENERAL HOSPITAL